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<b>(54) Title:</b> AN AUTOREGULATORY TETRACYCLINE-REGULATED SYSTEM FOR INDUCIBLE GENE EXPRESSION IN EU-CARYOTES		
<b>(57) Abstract</b>  <p>A tetracycline-regulated system which provides autoregulatory, inducible gene expression in cultured cells and transgenic animals is described. In the autoregulatory plasmid pTet-tTAk, a modified tTA gene called tTAk was placed under the control of Tetp. Tetracycline prevents tTA from binding to Tetp, preventing expression of both tTA and luciferase. This negative feedback cycle ensures that little or no tTA is produced in the presence of tetracycline, thereby reducing or eliminating possible toxic effects. When tetracycline is removed, however, this strategy predicts that tiny amounts of tTA protein (which may result from the leakiness of the minimal promoter), will bind to Tet-op and stimulate expression of the tTAk gene. A positive feedforward loop is initiated which in turn leads to higher levels of expression of tTA and thus, luciferase. Polynucleotide molecules encoding the autoregulatory system, as well as methods of enhancing or decreasing the expression of desired genes, and kits for carrying out these methods are described.</p>		

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# **An Autoregulatory Tetracycline-Regulated System For Inducible Gene Expression In Eucaryotes**

## ***Background of the Invention***

### ***Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development***

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

### ***Field of the Invention***

The invention is related to the recombinant DNA technology. A tetracycline-regulated system which provides autoregulatory, inducible gene expression in cultured cells and transgenic animals is described.

### ***Related Art***

Systems for inducible mammalian gene expression have typically encountered limitations such as basal leakiness, toxic or nonspecific effects of inducing agents or treatments, limited cell type applicability and low levels of expression (reviewed in Yarranton, G. T., *Curr. Opin. Biotech.* 3:506-511 (1992)). Recently, a system was described (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992)) that overcomes many of these difficulties by placing target genes under the control of a regulatory sequence (*tetO*) from the tetracycline-resistance operon of Tn10. In bacteria, this short sequence is bound tightly by the tetracycline repressor protein (*tetR*), and binding is blocked by the antibiotic tetracycline (Hillen, W. & Wissmann, A., in *Protein-Nucleic Acid Interaction, Topics in Molecular and Structural Biology*, Saenger, W. &

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Heinemann, U., eds., Macmillan, London (1989), pp. 143-162). A hybrid fusion protein, the tetracycline transactivator (tTA), combines the *tetR* DNA binding domain with the transcriptional activation domain of VP-16, such that when tTA binds to a minimal promoter containing *tetO* sequences, transcription of the target gene is activated. Tetracycline binding to tTA prevents activation presumably by causing a conformational change in the *tetR* portion of tTA which blocks binding of tTA to *tetO* (Hinrichs, W., *et al.*, *Science* 264:418-420 (1994)); gene activation is achieved by removing tetracycline (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992)).

The primary limitation of this system is difficulty in expressing even moderate levels of the tTA protein (undetectable by western blotting and barely detectable by gel electrophoresis mobility shift assay (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992))). Gossen and Bujard speculated that this was due to transcriptional "squenching" (Gill, G. & Ptashne, M., *Nature (London)* 334:721-724 (1988)) by the VP16 transactivator domain leading to death of cells expressing even modest levels of the tTA protein. These results combined with the observation of an apparently low level of expression of an inducible luciferase transgene using this system (Furth, P. A., *et al.*, *Proc. Natl. Acad. Sci. USA* 91:9302-9306 (1994)) suggest that inefficiencies in tTA expression may contribute to the difficulty.

### *Summary of the Invention*

By placing the tTA gene under the control of a promoter containing *tetO*, an autoregulatory tTA expression vector is created that allows high levels of tTA expression. It is demonstrated herein that this strategy permits the creation of highly inducible transfected cells with much greater efficiency than the constitutive system. Furthermore, it allows the creation of transgenic mice in which expression of a luciferase reporter gene can be controlled by altering the concentration of tetracycline in the drinking water of the animals. The

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autoregulated expression of transactivator protein should make the tetracycline system applicable to a wide array of problems requiring inducible mammalian gene expression.

5 The first embodiment of the invention relates to a composition of matter comprising a polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence. The open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is modified at its 10 5' end to provide an optimal context for translational initiation. In a preferred embodiment of the invention, it is modified to provide a unique restriction site, such as *HindIII*. In the most preferred embodiment of this invention, the open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is modified at its 5' end to encode an oligonucleotide identified as SEQ ID NO. -----. In a preferred embodiment, the polynucleotide molecule encoding a tetracycline transactivator fusion protein is DNA.

15 The second embodiment of the invention relates to a cloning vector containing the polynucleotide molecule of the invention. The most preferred embodiments of the invention relate to plasmids pTet-Splice and pTet-tTAK.

20 The third embodiment of the invention relates to a eucaryotic cell transfected with the polynucleotide molecule of the present invention. In a preferred embodiment, the eucaryotic cell contains tetracycline in an amount sufficient to suppress binding of tetracycline transactivator fusion protein to said inducible minimal promoter. In another preferred embodiment of the invention, 25 the eucaryotic cell is further transfected with a polynucleotide molecule encoding a heterologous protein operably linked to an inducible minimal promoter, which contains at least one *tet* operator sequence. In the most preferred embodiment of the invention, at least one of the polynucleotide molecules is operably linked to a minimal promoter and seven *tet* operator sequences. In a further preferred 30

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embodiment, the polynucleotide molecule encoding a tetracycline transactivator fusion protein is expressed in an amount sufficient to drive expression of the polynucleotide molecule, encoding the heterologous protein, in the absence of tetracycline. In another preferred embodiment, the tetracycline transactivator fusion protein is present in an amount sufficient to drive expression of the heterologous protein.

The fourth embodiment of the invention relates to a method to decrease or shut off expression of a heterologous protein comprising

(a) transforming a eucaryotic cell with

(i) a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence;

(ii) a second polynucleotide molecule encoding the heterologous protein, said protein being operably linked to an inducible minimal promoter, and said promoter containing at least one *tet* operator sequence; and

(b) cultivating the eucaryotic cell in a medium comprising tetracycline or a tetracycline analogue. In a preferred embodiment, the second polynucleotide molecule is operably linked to a minimal promoter and seven *tet* operator sequences.

The fifth embodiment of the invention relates to a method to activate or enhance the expression of a heterologous protein comprising

(a) transforming a eucaryotic cell with

(i) a first polynucleotide molecule encoding tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible promoter, which promoter contains at least one *tet* operator sequence;

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(ii) a second polynucleotide molecule encoding the heterologous protein, said protein being operably linked to an inducible minimal promoter, and said promoter containing at least one *tet* operator sequence; and

(b) cultivating the eucaryotic cell in a medium lacking tetracycline or a tetracycline analogue.

The sixth embodiment of the invention relates to a kit comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a procaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence; and a second container means contains a second polynucleotide molecule encoding said inducible minimal promoter, which promoter contains at least one *tet* operator sequence, which *tet* operator sequence is strategically positioned for being operably linked to a heterologous polynucleotide sequence encoding a polypeptide.

The seventh embodiment of the invention relates to a kit comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a eucaryotic cell transfected with a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a procaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence; and a second container means contains a second polynucleotide molecule comprising an inducible minimal promoter, which promoter contains at least one *tet* operator sequence, which *tet* operator sequence is strategically positioned for being operably linked to a heterologous polynucleotide sequence encoding a heterologous polypeptide.

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The polynucleotides described in this invention and cell lines containing said polynucleotides are research tools which allow one to tightly and quantitatively control the expression of a large variety of genes. This is of interest in broad areas of basic as well as applied research.

5           The invention also relates to the construction of eucaryotic production cell lines and strains in which the synthesis of the product, RNA or protein, is controlled by the *tet* regulatory system. These cell lines and strains allow one to induce protein synthesis at a predetermined time point or within a time window during a fermentation process. This control allows one to synthesize in large  
10       scale cultures gene products whose prolonged presence is lethal to the cells. Alternatively, the cells allow one to induce production of RNA when it is desirable to generate RNA molecules used to achieve a variety of cellular tasks, regulation, and function. Induction of RNA production can be controlled where, for example, the RNA are used as antisense oligos to inhibit the function of a  
15       gene which is either homologous or heterologous to the cell.

          The invention also relates to the construction of cell lines which can be used in screening systems to identify compounds of pharmaceutical or other commercial value. In such systems, the expression of target molecules including but not limited to receptors such as the GABA or estrogen receptor, whose long  
20       term presence, in particular, in high copy numbers is often cell damaging, can be temporarily and quantitatively controlled.

          The invention also relates to the construction of transgenic animals in which the expression of a single gene can be controlled externally by the *tet* regulatory system. Such genes include human genes whose expression, failure  
25       of expression, or other defects are involved in human diseases. Such transgenic animals can serve as models for human diseases in therapeutic studies and for the screening of compounds of pharmaceutical interest. The invention also relates to the construction of transgenic animals for the production of compounds of pharmaceutical or other commercial interest.

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Another important application of this system makes possible the temporal control of gene expression, where for example, the gene of interest is introduced into a cell, animal, or plant to compensate for lethal knock out of certain genes in the transgene. Using the system to introduce a copy of the gene which has been suppressed or deactivated, enough protein or RNA can be produced to allow growth and development of the cell or the plant or animal until a time at which it is desired to shut off production of the gene and carry out the manipulations that require the lethal knock out of said gene.

### *Brief Description of the Figures*

**Figure 1** is a schematic representation depicting the autoregulatory strategy for inducible gene expression. Autoregulatory expression of tTA is accomplished in pTet-tTA by placing the tTA gene (white box) under the control of Tetp consisting of seven copies of the tetracycline operator sequence (Tet-op; dark shaded box) upstream of the minimal human cytomegalovirus (hCMV) promoter region containing a TATA box and transcription start site (black circle). The luciferase reporter gene (shaded box) of pUHC13-3 is also controlled by the Tetp promoter. The tTA protein is shown as two adjoining striped boxes to represent the two domains of the protein (for DNA binding and transactivation). In the presence of tetracycline (left panel), the basal activity of the minimal hCMV promoter results in expression of very low levels of the tTA protein (represented as a small tTA icon), and any tTA protein produced is blocked from binding to Tet-op. Both luciferase and tTA expression are therefore maintained at low levels (thin, short dashed lines). When tetracycline is removed (right panel), the small amounts of tTA present bind Tet-op, stimulating expression of the tTA gene. Higher levels of the tTA protein now stimulate higher levels of tTA and thus, luciferase expression (heavy, long dashed lines).

**Figures 2A and 2B** are bar graphs which depict inducible V(D)J recombination in NIH3T3 fibroblasts.

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**Figure 2A** depicts a bar graph analysis of clones containing pcDNA-tTAk (constitutive tTA expression). Seventeen stable transfectant clones (S1-1 to S1-17) were derived and assayed for the ability to carry out V(D)J recombination by transient co-transfection with a V(D)J recombination substrate and Tetp-controlled RAG-1 and RAG-2 expression vectors. Parallel transfections were performed in the presence (Tet+) and absence (Tet-) of tetracycline in the growth media, and the V(D)J recombination frequency (expressed as a percent) was determined as described in Materials and Methods. For comparison, four control assays performed in NIH3T3 cells are also shown (first two samples): transfection of the recombination substrate in the absence of RAG-1 and RAG-2, with and without tetracycline, and co-transfection of the recombination substrate with constitutive RAG expression vectors, with and without tetracycline. Tetracycline had no effect on V(D)J recombination frequency when RAG-1 and RAG-2 were expressed from constitutive hCMV promoters. Fold induction achieved by removing tetracycline is indicated above the bars in cases where clearly detectable recombination was observed.

**Figure 2B** depicts a bar graph analysis of clones containing pTet-tTAk (autoregulatory tTA expression). Ten stable transfectant clones (S2-1 to S2-10) and two clones containing pTet-tTAk, pTet-R1A/C and pTet-R2A (S4-9 and S4-5) were assayed for the ability to carry out V(D)J recombination as described above. The first two samples are the same control samples described in Fig. 2A. Note the difference in the recombination frequency axis scale between Fig. 2A and Fig. 2B. Asterisks (\*) mark two Tet+ transfections that yielded very small numbers of ampicillin resistant colonies, making the calculated recombination frequency unreliable. Consequently, the fold-inducibility for these clones is not shown. The number of ampicillin resistant colonies was low in these experiments (range, 350-55,550). Based on additional assays on some of the cell lines, we estimate that the reported recombination frequencies are as much as two fold overestimates in both Fig. 2A and Fig. 2B.

**Figures 3A, 3B, and 3C** are photographs of RNA blots and a Western blot depicting the detection of mRNA and protein expression activated using the inducible, autoregulatory system.

**Figure 3A** is a photograph of a RNA blot of total cell RNA from S4-9 (stable co-transfectant of pTet-tTak, pTet-R1A/C, and pTet-R2A) cultured 23h in the presence or absence of tetracycline, and S2-6 (stable transfectant of pTet-tTak) transiently transfected with either pTet-R1 or pTet-R2 and cultured for 48 hours in the presence or absence of tetracycline. Blots were sequentially hybridized with probes detecting tTak, RAG-1 and/or RAG-2, and  $\gamma$ -actin mRNA.

**Figure 3B** is a photograph of a Western blot of cell extracts from S2-6 cells cultured for 48 hours in the presence or absence of tetracycline. Blot was probed with anti-*tet* R antibody-containing hybridoma supernatant which detects the tTA protein. The dye front is indicated.

**Figure 3C** is a photograph of a blot of total cell RNA from thymus (T) and lung (L) of pTet-tTak/Tet-luciferase transgenic mice maintained for 7 days in the presence or absence of tetracycline in their drinking water. Approximately 20  $\mu$ g of RNA was loaded per lane.

**Figure 4** is a graph depicting inducible luciferase activity in tissues of transgenic mice. Values represent the relative light units (rLU) (with lysis buffer background subtracted) per mg protein in tissue lysates from 4-7 week old mice maintained for 7-8 days in the presence or absence of tetracycline in their drinking water. Open triangles are transgene negative mice; open circles are uninduced transgene positive mice; and closed circles are induced transgene positive mice. Mice were genetically identical with respect to the transgenes. Results are compiled from three separate experiments.

**Figure 5** is a graph depicting the ability of pTet-tTak to induce expression of luciferase activity in a transfected fibroblast cell line. pUHC13-3 was co-transfected with either pTet-tTak or pcDNA-tTak into NIH3T3 fibroblast cells, and 48 hours later the cells were harvested and the luciferase light

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units present in the extracts was measured. pTet-tTAk transfections were performed in the presence and absence of tetracycline (tet), while pcDNA-tTAk transfection was performed only in the absence of tet. It is important to note that the luciferase values have not been corrected for transfection efficiency. In addition, under the conditions used, pcDNA-tTAk would replicate inside the cells while pTet-tTAk would not. Therefore, comparisons between the values obtained with pTet-tTAk and pcDNA-tTAk are not meaningful.

**Figure 6** is a photograph of an autoradiograph depicting loss of tTA protein at 16 days without tetracycline in S2-6 cells. **Figure 6** is a photograph of a Western blot of cell extracts from cultured S2-6 and S2-1 cell lines. First lane: marker proteins (no bands, visible); second and third lanes: S2-6 cells grown in the absence of tet for 16 days; fourth and fifth lanes: S2-6 cells grown in the presence of 0.5 µg/ml tet for 16 days; sixth lane: S2-1 cells grown in the presence of 0.5 µg/ml tet for 16 days; and seventh lane: S2-6 cells grown in the absence of tet for 2 days. A signal for the tTA protein is seen in S2-6 cells grown in the absence of tet for 2 days, but not in the same cells cultured in the absence of tet for 16 days. The band seen at the bottom in lanes 2-7 ("protein front") is the dye front and represents a non-specific signal.

**Figure 7** is a schematic depiction of the construction of pCMV-tTAk, pcDNAI-neo, and pcDNA-tTAk.

**Figure 8** is a schematic depiction of the construction of pTet-Splice and pTet-tTAk.

**Figure 9A** depicts a restriction map of pTet-Splice. Cloning sites are shown in boldface print. Note that there are two *EcoRI* sites.

**Figures 9B-9G** depict the nucleotide sequence of pTet-Splice (SEQ ID NO ---).

**Figure 10A** depicts a restriction map of pTet-tTAk.

**Figures 10B-10G** depict the nucleotide as well as partial amino acid sequence of pTet-tTAk (SEQ ID NO ---).

**Figure 11A** depicts a restriction map of pUHD15-1.

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Figures 11B-11F depict the nucleotide as well as partial amino acid sequence of pUHD15-1.

Figure 12A depicts a restriction map of pUHC-13-3.

Figures 12B-12F depict the nucleotide as well as partial amino acid sequence of pUHC-13-3 (SEQ ID NO ---).

### *Detailed Description of the Preferred Embodiments*

A system for tetracycline-regulated inducible gene expression was described recently which relies on constitutive expression of a transactivator fusion protein (tTA) consisting of the DNA binding domain of the tetracycline repressor and the transcriptional activation domain of VP16 ( U.S. patent application serial number 08/076,726, herein incorporated by reference in its entirety; Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992)). This system yielded only low levels of transactivator protein, probably because tTA is toxic. To avoid this difficulty, the tTA gene was placed under the control of the inducible promoter to which tTA binds, making expression of tTA itself inducible and autoregulatory.

When used to drive expression of the recombination activating genes RAG-1 and RAG-2, the autoregulatory system yielded both substantially higher levels of V(D)J recombination activity (70 fold on average) and inducible expression in a much larger fraction of transfected cells (autoregulatory, 90% vs. constitutive, 18%). In addition, this system allowed the creation of inducible transgenic mice in which expression of a luciferase transgene was induced tens to hundreds fold the basal levels in most tissues examined. Induced levels of expression were highest in thymus and lung and appear to be substantially higher than in previously reported inducible luciferase transgenic mice created with the constitutive system. With the modified system, inducible transactivator mRNA and protein were easily detected in cell lines by RNA and western blotting, and transactivator mRNA was detected by RNA blotting in some tissues of transgenic

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mice. This autoregulatory system represents an improved strategy for tetracycline regulated gene expression both in cultured cells and in transgenic animals.

As mentioned above, the inducible tetracycline expression system described recently (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992)) relies on constitutive expression of the tTA gene from a fully functional human cytomegalovirus (hCMV) promoter, and a luciferase reporter gene under the control of the inducible promoter Tetp. In this system, tetracycline prevents the activation of luciferase gene expression, but does not prevent the tTA protein from exerting potentially deleterious effects on cells (Gill, G. & Ptashne, M., *Nature (London)* 334:721-724 (1988)).

Hence, in the autoregulatory plasmid pTet-tTAk, a modified tTA gene called tTAk was placed under the control of Tetp (Figure 1). Tetracycline prevents tTA from binding to Tetp, preventing expression of both tTA and luciferase. This negative feedback cycle ensures that little or no tTA is produced in the presence of tetracycline, thereby reducing or eliminating possible toxic effects. When tetracycline is removed, however, this strategy predicts that tiny amounts of tTA protein (which may result from the leakiness of the minimal promoter), will bind to Tet-op and stimulate expression of the tTAk gene. A positive feedforward loop is initiated which in turn leads to higher levels of expression of tTA and thus, luciferase (Figure 1). For constitutive expression of tTA, the tTAk gene was placed under the control of the hCMV promoter, followed with additional sequences to direct RNA splicing and polyadenylation of the tTA transcript. This plasmid (pcDNA-tTAk) also includes the neo gene, which allows for selection of the plasmid in mammalian cells.

The present invention relates to an autoregulatory control system that in eucaryotic cells allows regulation of expression of an individual gene over 200 to 3700 fold. This system is based on regulatory elements of a tetracycline-resistance operon, e.g. Tn10 of *E. coli* (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162), in which

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transcription of resistance-mediating genes is negatively regulated by a tetracycline repressor (*tetR*). In the presence of tetracycline or a tetracycline analogue, *tetR* does not bind to its operators located within the promoter region of the operon and allows transcription. By combining *tetR* with a protein domain capable of activating transcription in eucaryotes, such as (i) acidic domains (e.g. the C-terminal domain of VP16 from HSV (Triezenberg *et al.*, *Genes Dev.* 2:718-729 (1988)) or empirically determined, non-eucaryotic acidic domains identified by genetic means (Giniger and Ptashne, *Nature* 330:670-672 (1987))) or (ii) proline rich domains (e.g. that of CTF/NF-1 (Mermoud *et al.*, *Cell* 58:741-753 (1989))) or (iii) serine/threonine rich domains (e.g. that of Oct-2 (Tanaka and Herr, *Cell* 60:375-386 (1990))) or (iv) glutamine rich domains (e.g. that of Sp1 (Courey and Tjian, *Cell* 55:867-898 (1988))) a hybrid transactivator is generated that stimulates minimal promoters fused to tetracycline operator (*tetO*) sequences. These promoters are virtually silent in the presence of low concentrations of tetracycline, which prevents the tetracycline-controlled transactivator (tTA) from binding to *tetO* sequences.

The specificity of the *tetR* for its operator sequence (Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saeger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162) as well as the high affinity of tetracycline for *tetR* (Takahashi *et al.*, *J. Mol. Biol.* 187:341-348 (1986)) and the well-studied chemical and physiological properties of tetracyclines constitute a basis for an autoregulatory inducible expression system in eucaryotic cells far superior to the *lacR/O/IPTG* system.

In particular, the invention relates to a first polynucleotide molecule coding for a transactivator fusion protein comprising the *tet* repressor (*tetR*) and a protein domain capable of activating transcription in eucaryotes, wherein the first polynucleotide molecule is operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence. The polynucleotide coding for *tetR* may be obtained according to Postle *et al.*, *Nucl.*

*Acids Res.* 12:4849-4863 (1984), the contents of which are fully incorporated by reference herein. Other *tetR* sequences and the respective binding sites for these repressors are identified (Waters *et al.*, *Nucl. Acids Res.* 11:6089-6105 (1983); Postle *et al.*, *Nucl. Acids Res.* 12:4849-4863 (1984); Unger *et al.*, *Gene* 31:103-108 (1984); Unger *et al.*, *Nucl. Acids Res.* 12:7693-7703 (1984); Tovar *et al.*, *Mol. Gen. Genet.* 215:76-80 (1988); for comparison and overview see Hillen and Wissmann in *Protein-Nucleic Acid Interaction*, Topics in Molecular and Structural Biology, Saenger and Heinemann (eds.), Macmillan, London, Vol. 10, pp. 143-162 (1989)) and can also be utilized for the expression system described.

The polynucleotide coding for the negatively charged C-terminal domain of HSV-16, a protein known to be a powerful transcription transactivator in eucaryotes, may be obtained according to Triezenberg *et al.*, *Genes Dev.* 2:718-729 (1988), the contents of which are fully incorporated by reference herein. Preferably, the activating domain comprises the C-terminal 130 amino acids of the virion protein 16.

The polynucleotide molecule coding for *tetR* may be linked to a polynucleotide molecule coding for the activating domain of HSV-16 and recombined with vector DNA in accordance with conventional techniques, including blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases.

The *tetO* sequence may be obtained, for example, according to Hillen & Wissmann, "Topics in Molecular and Structural Biology," in *Protein-Nucleic Acid Interaction*, Saenger & Heinemann, eds., Macmillan, London, 1989, Vol. 10, pp. 143-162, the contents of which are fully incorporated by reference herein. Other *tetO* sequences which may be used in the practice of the invention may be obtained from the references given in the following (Waters *et al.*, *Nucl. Acids Res.* 11:6089-6105 (1983); Postle *et al.*, *Nucl. Acids Res.* 12:4849-4863 (1984);

Unger *et al.*, *Gene* 31:103-108 (1984); Unger *et al.*, *Nucl. Acids Res.* 12:7693-

7703 (1984); Tovar *et al.*, *Mol. Gen. Genet.* 215:76-80 (1988); for comparison and overview see Hillen and Wissmann in *Protein-Nucleic Acid Interaction*, Topics in Molecular and Structural Biology, Saenger and Heinemann (eds.), Macmillan, London, Vol. 10, pp. 143-162 (1989)), the disclosures of which are fully incorporated by reference herein in their entirety. One, two, three, four, five, six, seven, eight, nine or ten or more copies of the *tet* operator sequence may be employed, with a greater number of such sequences allowing an enhanced range of regulation. Multiple copies of the *tet* operator sequence provides a synergistic effect on the ability to control expression of the heterologous protein.

The polynucleotide sequence specifying the cytomegalovirus promoter may be obtained according to Boshart *et al.*, *Cell* 41:521-530 (1985), the contents of which are fully incorporated by reference herein. Preferably, positions +75 to -53 or +75 to -31 of the promoter-enhancer may be employed. The promoter may be followed by a polylinker and then by the gene coding for the tetracycline transactivator fusion protein.

The invention also relates to an autoregulatory tetracycline-regulated system for inducing gene expression in eucaryotes, wherein a second polynucleotide molecule is introduced into the host. The second polynucleotide molecule encodes a protein of interest, wherein said polynucleotide is operably linked to a minimal promoter operatively linked to at least one *tet* operator (*tetO*) sequence. The minimal promoter linked to at least one *tetO* sequence is obtained as described above with regard to the first polynucleotide molecule. The difference between the first and the second polynucleotide molecules is that the promoter may be followed by a polylinker and then by the gene encoding the protein of interest. While the luciferase gene or other reporter genes may be used to demonstrate the operability of the regulatory system, the invention is not intended to be so limited.

The invention further relates to homologous and heterologous genes involved in developmental and differentiation processes, as well as in metabolic

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pathways ensuring cellular function and communication. It relates furthermore to cellular systems utilized in the production of substances of commercial interest, including, but not limited to immunoglobulins, components of the cytoskeleton, cell adhesion proteins, receptors, cytokines peptide hormones and enzymes.

5           The present invention also relates to eucaryotic cells transfected with the polynucleotide molecules of the present invention. In particular, the invention relates to eucaryotic cells transfected with

          (a)     a first polynucleotide molecule coding for a transactivator fusion protein comprising a prokaryotic *tet* repressor and a protein capable of activating  
10           transcription in eucaryotes, wherein said first polynucleotide molecule is operably linked to a minimal promoter and at least one *tet* operator sequence; and

          (b)     a second polynucleotide molecule coding for a protein, wherein said second polynucleotide molecule is operably linked to a minimal promoter and at least one *tet* operator sequence.

15           The two polynucleotide molecules may reside on the same or separate vectors. In a preferred embodiment, the first polynucleotide is integrated into the chromosome of a eucaryotic cell or transgenic animal and the second polynucleotide is introduced as part of a vector. Integration may be achieved where there is crossover at regions of homology shared between the incoming  
20           polynucleotide molecule and the particular genome.

          The expression of the heterologous protein from such transfected eucaryotic cells may be tightly regulated. Unexpectedly, it has been determined that the autoregulatory expression system of the present invention may be used to induce expression by greater than 200 to 3700 fold, compared to greater than  
25           50 to 100 fold increase observed when the constitutive expression system is used. In addition, it has been discovered that the expression system of the present invention allows one to rapidly turn on and off the expression of the heterologous gene in a reversible way. Moreover, it has been discovered that the expression system of the invention allows one to achieve a desired level of expression  
30           according to how much tetracycline or tetracycline analogue is employed. Thus,

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the autoregulatory expression system of the present invention is a great advance in the art.

The invention also relates to a method to decrease or to shut off (deactivate) the expression of a protein coded for by a polynucleotide, comprising cultivating the transfected eucaryotic cells of the present invention in a medium comprising tetracycline or a tetracycline analogue. It is possible to closely control the extent of expression by carefully controlling the concentration of tetracycline or tetracycline analogue in the culture media. As little as 0.0001  $\mu\text{g/ml}$  of tetracycline will begin to result in a decrease of polypeptide (luciferase) expression. At about 0.1-1.0  $\mu\text{g/ml}$  the expression is essentially shut off. The concentration of tetracycline or tetracycline analog which can be used to regulate the expression level may range from about 0.0001 to about 1  $\mu\text{g/ml}$ .

The invention also relates to a method to turn on (activate) or to increase the expression of a protein coded for by a polynucleotide, comprising cultivating the eucaryotic cell of the invention in a medium lacking tetracycline or a tetracycline analogue.

Media which may be used in the practice of the invention include any media which are compatible with the transfected eucaryotic cells of the present invention. Such media are commercially available (Gibco/BRL).

The invention also relates to transgenic animals comprising one or two of the polynucleotide molecules of the present invention. Such transgenic animals may be obtained, for example, by injecting the polynucleotide into a fertilized egg which is allowed to develop into an adult animal. In particular, a few hundred DNA molecules are injected into the pro-nucleus of a fertilized one cell egg. The microinjected eggs are then transferred into the oviducts of pseudopregnant foster mothers and allowed to develop. It has been reported by Brinster *et al.*, *Proc. Natl. Acad. Sci. USA* 82:4438-4442 (1985), the contents of which are fully incorporated by reference herein, that about 25% of mice which develop will inherit one or more copies of the microinjected DNA. Alternatively, the transgenic animals may be obtained by utilizing recombinant ES cells for the

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generation of the transgenes, as described by Gossler *et al.*, *Proc. Natl. Acad. Sci. USA* 83:9065-9069 (1986), the contents of which are fully incorporated by reference herein. Animals transgenic for the gene encoding a *tetR*/transcriptional activator domain fusion protein under the transcriptional control of at least one Tet-op sequences described above and/or the gene under control of this regulatory protein can be generated e.g. by the coinjection of the two polynucleotide molecules. Alternatively, independent animal lines transgenic for only one of the polynucleotides described can be generated in a first step:

(i) Animals transgenic only for the gene encoding the desired heterologous protein to be controlled by the transactivator can be screened for the desired nonactivated expression level. This includes indicator animals transgenic for a reporter gene (e.g. *cat*, *luc*, *lacZ*) under transcriptional control of the *tetR*/transcriptional activator domain fusion protein dependent minimal promoter, which are easy to screen for integration sites showing the desired, in general a low level basal expression. If advantageous, these empirically determined loci can be used subsequently for a homologous recombination approach (Mansour *et al.*, *Nature* 336:348-352 (1988)), by which the reporter gene is substituted by a respective gene of interest in the previously analyzed integration site.

(ii) Animals transgenic only for a gene encoding a *tetR*/transcriptional activator domain fusion protein can be analyzed for the desired expression pattern of the regulator protein.

Subsequently, the desired double transgenic animals are obtained by breeding the two complementary transgenic animal lines.

### ***The Definitions***

In the description that follows, a number of terms used in recombinant DNA technology are utilized extensively. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

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**Expression:** Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

**Autoregulatory expression vector:** It refers to the invention as described herein. A modified tTA gene called tTAk is placed under the control of Tetp (Figure 1). Tetracycline prevents tTA from binding to Tetp, preventing expression of both tTA and thus the desired protein (such as luciferase in Figure 1). This negative feedback cycle ensures that little or no tTA is produced in the presence of tetracycline, thereby reducing or eliminating possible toxic effects. When tetracycline is removed, however, this strategy predicts that tiny amounts of tTA protein (which may result from the leakiness of the minimal promoter), will bind to Tet-op and stimulate expression of the tTAk gene. A positive feedforward loop is initiated which in turn leads to higher levels of expression of tTA and thus, luciferase (Figure 1).

**Optimal context for translational initiation:** consists of the ATG methionine initiation codon, plus flanking nucleotides as defined by: Kozak, M., *Cell* 44:283-292 (1986). The sequence is: CC(A or G)CCATGG, with the initiation codon shown in bold. This sequence provides for the most efficient initiation of translation by the translation machinery.

**Promoter:** A DNA sequence generally described as the region 5' of a gene, located proximal to the start site of transcription. The transcription of an adjacent gene(s) is initiated at the promoter region. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

**Inducible Minimal Promoter:** It refers to the minimum number of nucleic acids from a promoter sequence, which in combination with other regulatory elements, is capable of initiating transcription. A minimal promoter, at the minimum, defines the transcription start site but by itself is not capable, if at all, of initiating transcription efficiently. The activity of such minimal

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promoters depend on the binding of activators such as a tetracycline-controlled transactivator to operably link binding sites.

**V(D)J Recombination:** So called for the variable (V), diversity (D), and joining (J) gene segments used in recombination, it is a process by which the developing lymphocytes begin to generate their enormous range of binding specificities from a limited amount of genetic information. It is known to assemble seven different loci in developing lymphocytes:  $\mu$ ,  $\kappa$ , and  $\lambda$  in B cells, and  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  in T cells (for reviews see Blackwell and Alt (1988), *Immunoglobulin genes*, In *Molecular Immunology*, Hames and Glover, eds. (Washington, D.C.: IRL Press), pp. 1-60; Davis and Bjorkman, *Nature* 334:395-402 (1988); Raulet, D.H., *Annu. Rev. Immunol.* 7:175-207 (1989)).

**RAG-1 and RAG-2:** RAG-1 (recombination activating gene-1) and RAG-2 are genes co-expressed in maturing lymphocytes. Expression of both genes is absolutely required for V(D)J recombination and lymphocyte development. When transfected together into fibroblasts, RAG-1 and RAG-2 induce V(D)J recombination activity. The RAG-1 and RAG-2 genes lie adjacent to each other in the vertebrate genome and encode unrelated proteins. Both RAG-1 and RAG-2 are conserved between species that carry out V(D)J recombination, and their expression pattern correlates precisely with that of V(D)J recombinase activity.

**Founder:** It is the original (first generation) transgenic animal, *i.e.* an animal carrying a transgene, which has been made by manipulating the genome of a fertilized egg and implanting the egg into a pseudopregnant animal.

**Operator:** It is the site on DNA at which a repressor protein binds to prevent transcription from initiating at the adjacent promoter.

**Operon:** is a unit of bacterial gene expression and regulation, including structural genes and control elements in DNA recognized by regulator gene product(s).

**Repressor:** It is a protein that binds to operator on DNA or to RNA to prevent transcription or translation, respectively.

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**Repression:** is the ability of an organism to prevent synthesis of certain enzymes when their products are present: more generally, refers to inhibition of transcription (or translation) by binding of repressor protein to specific site on DNA (or mRNA).

5       **Open Reading Frame (ORF):** contains a series of triplets coding for amino acids without any termination codons; sequence is (potentially) translatable into protein.

**Heterologous Protein:** is a protein that does not naturally occur in the specific host organism in which it is present.

10       **Unique Restriction site:** refers to a single occurrence of a site on the nucleic acid that is recognized by a restriction enzyme.

15       **Tetracycline Transactivator Fusion Protein:** A hybrid fusion protein, the tetracycline transactivator (tTA), combines the *tetR* DNA binding domain with the transcriptional activation domain of VP-16, such that when tTA binds to a minimal promoter containing *tetO* sequences, transcription of the target gene is activated. Tetracycline binding to tTA prevents activation presumably by causing a conformational change in the *tetR* portion of tTA which blocks binding of tTA to *tetO* (Hinrichs, W., *et al.*, *Science* 264:418-420 (1994)); gene activation is achieved by removing tetracycline (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992)).

20       **Domain:** of a protein is a discrete continuous part of the amino acid sequence that can be equated with a particular function.

25       **Cloning vector:** A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector.

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**Expression vector:** A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Promoter sequences may be either constitutive or inducible.

**Eucaryotic Cell:** According to the invention, a eucaryotic cell may be a cell of any eucaryotic organism including, but not limited to, yeast, plant cells, insect cells, e.g. Schneider and Sf9 cells; mammalian cells, e.g. lymphoid and HeLa cells (human), NIH3T3 and embryonic stem cells (murine), and RK13 (rabbit) cells.

**Recombinant Eucaryotic Host:** According to the invention, a recombinant eucaryotic host may be any eucaryotic cell which contains the polynucleotide molecules of the present invention on an expression vector or cloning vector. This term is also meant to include those eucaryotic cells that have been genetically engineered to contain the desired polynucleotide molecules in the chromosome, genome or episome of that organism. Thus, the recombinant eucaryotic host cells are capable of stably or transiently expressing the proteins.

**Recombinant vector:** Any cloning vector or expression vector which contains the polynucleotide molecules of the invention.

**Host:** Any prokaryotic or eucaryotic cell that is the recipient of a replicable vector. A "host," as the term is used herein, also includes prokaryotic or eucaryotic cells that can be genetically engineered by well known techniques to contain desired gene(s) on its chromosome or genome. For examples of such hosts, see Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

**Gene:** A DNA sequence that contains information needed for expressing a polypeptide or RNA molecule, including an RNA molecule which is *not* translated into polypeptide and functions as RNA, e.g., ribosomal genes.

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**Structural gene:** A DNA sequence that is transcribed into messenger RNA (mRNA) that is then translated into a sequence of amino acids characteristic of a specific polypeptide.

5 **Polynucleotide molecules:** A polynucleotide molecule may be a polydeoxyribonucleic acid molecule (DNA) or a polyribonucleic acid molecule (RNA).

**Complementary DNA (cDNA):** A "complementary DNA," or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA and from which intervening sequences (introns) have been removed.

10 **Fragment:** A "fragment" of a polypeptide or a polynucleotide molecule is meant to refer to any polypeptide or polynucleotide subset of that molecule.

15 **Tetracycline Analogue:** A "tetracycline analogue" is any one of a number of compounds that are closely related to tetracycline and which bind to the *tet* repressor with a  $K_a$  of at least about  $10^6 \text{ M}^{-1}$ . Preferably, the tetracycline analogue binds with an affinity of about  $10^9 \text{ M}^{-1}$  or greater, e.g.  $10^{11} \text{ M}^{-1}$ . Examples of such tetracycline analogues include, but are not limited to those disclosed by Hlavka and Boothe, "The Tetracyclines," in *Handbook of Experimental Pharmacology* 78, R.K. Blackwood *et al.* (eds.), Springer-Verlag, Berlin-New York, 1985; L.A. Mitscher, "The Chemistry of the Tetracycline Antibiotics," *Medicinal Research* 20 9, Dekker, New York, 1978; Noyee Development Corporation, "Tetracycline Manufacturing Processes," *Chemical Process Reviews*, Park Ridge, N.J., 2 volumes, 1969; R.C. Evans, "The Technology of the Tetracyclines," *Biochemical Reference Series 1*, Quadrangle Press, New York, 1968; and H.F. Dowling, "Tetracycline," *Antibiotics Monographs*, no. 3, Medical Encyclopedia, New York, 1955; the contents of each of which are fully incorporated by reference 25 herein.

***Comparison of the constitutive and autoregulatory inducible expression systems in cultured cells***

After confirming that pcDNA-tTAK and pTet-tTAK could direct high levels of expression of luciferase activity, and that expression directed by pTet-tTAK was inducible (*see* Figure 5), the functional properties of these plasmids were compared in a more stringent assay: the ability to express high levels of the proteins encoded by the recombination activating genes RAG-1 and RAG-2 (Schatz, D. G. *et al.*, *Cell* 59:1035-1048 (1989); Oettinger, M. A. *et al.*, *Science* 248:1517-1523 (1990)). During lymphoid development, RAG-1 and RAG-2 participate in the assembly of functional immunoglobulin and T cell receptor genes from component variable (V), diversity (D) and joining (J) gene segments, a process known as V(D)J recombination. Most important for the experiments described here, RAG-1 and RAG-2 are necessary and sufficient to activate the V(D)J recombinase in non-lymphoid cells (reviewed in Schatz, D. G. *et al.*, *Annu. Rev. Immunol.* 10:359-383 (1992)), and the activity of the V(D)J recombinase can be quantitatively assayed using extrachromosomal recombination substrates (Hesse, J. E. *et al.*, *Cell* 49:775-783 (1987)).

Extensive efforts to express RAG-1 and RAG-2 in NIH3T3 fibroblast cells using a variety of promoters have revealed that it is difficult to achieve a recombination frequency (Rn) of greater than a few percent, as assayed with standard extrachromosomal recombination substrates (Sadofsky, *et al.*, *Nuc. Acids. Res.* 22:1805-1809 (1994); Sadofsky, *et al.*, *Nuc. Acids. Res.* 21:5644-5650 (1993); Cuomo and Oettinger, *Nuc. Acids. Res.* 22:1810-1814 (1994)). Only high titer RAG-retroviruses developed by others have reproducibly shown the ability to achieve an Rn as high as 10% (Silver, D. P. *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6100-6104 (1993)). What is clear, however, is that Rn correlates strongly with RAG expression levels over at least three orders of magnitude (Rn from 0.01% to well above 10%; Oltz, E. M., *et al.*, *Mol. Cell. Biol.* 13(10):6223-6230 (1993)). Thus the ability to express the RAG proteins, as measured by

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V(D)J recombinase activity, is an appropriate test of an inducible expression system both because of the difficulties that have been encountered in expressing the proteins and because of the sensitivity and range of the assay.

NIH3T3 fibroblast clones stably transfected with pcDNA-tTAk (17 clones) or with pTet-tTAk (10 clones) were tested for their ability to perform V(D)J recombination after transient transfection with a recombination substrate and Tetp-regulated RAG-1 and RAG-2 (Figure 2). Each clone was assayed in parallel in the presence (uninduced state) or absence (induced state) of tetracycline and the results compared to control transfections either lacking RAG-1 or RAG-2 (first sample in Figures 2A and 2B) or containing highly active, constitutive RAG expression constructs (in which RAG expression is driven by the hCMV promoter; second sample in Figures 2A and 2B). In addition, two NIH3T3 clones stably transfected with pTet-tTAk and the Tetp-regulated RAG expression vectors were assayed by transient transfection of the recombination substrate in the presence or absence of tetracycline (last two samples in Figure 2B).

The autoregulatory expression system (pTet-tTAk) represents a substantial improvement over the constitutive expression system (pcDNA-tTAk). Only 3 of 17 (18%) pcDNA-tTAk transfectants had clearly detectable levels of V(D)J recombination (Figure 2A), with the highest levels of recombination (in clone S1-17) being 3 fold that seen in the positive control with constitutively active RAG expression vectors (second sample; Figure 2A). Removal of tetracycline induced recombination in these three clones (S1-12, S1-13, S1-17) by greater than 50 to 100 fold. In contrast, 9 of 10 (90%) pTet-tTAk transfectants (Figure 2B) showed high levels of recombination (note the difference in scale between Figures 2A and 2B), with the highest levels (28% in S2-1) being nearly 50 fold higher than the positive control (Figure 2B, second sample). Inducibility in these nine clones was excellent, ranging from over 200 fold to 3700 fold. Equally high were the observed recombination frequencies achieved in clones

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stably transfected with pTet-tTAk and Tetp-regulated RAG plasmids (S4-9 and S4-5; last two samples in Figure 2B).

Further characterization of the pTet-tTAk transfectant S2-6 and the pTet-tTAk + pTet-R1A/C + pTet-R2A transfectant S4-9 demonstrated that the ability to induce high levels of V(D)J recombinase activity is reproducible and that recombination decreases three fold with 0.01 µg/ml tetracycline and twenty fold with 0.1 µg/ml tetracycline. Greater than 50% cell death was observed within 10 days and a loss of detectable tTA protein was detected by 3 weeks in S2-6 cells cultured in the absence of tetracycline (Figure 6).

Figure 3A demonstrates that mRNA corresponding to the tTAk and RAG-1 and RAG-2 genes is detected in induced cell lines stably expressing tTA and stably or transiently expressing RAG-1 and/or RAG-2. Figure 3B shows that in S2-6 cells induced for 48 hours by tetracycline removal, tTA protein is easily detectable by Western blotting.

### *The creation of inducible transgenic mice*

To assess the potential of using the autoregulatory tetracycline system in transgenic mice, the relevant portions of pTet-tTAk and pUHC13-3 were purified and co-microinjected into fertilized eggs, which were then implanted into pseudopregnant female mice. Five transgene positive founders were screened for inducibility by measurement of luciferase levels in peripheral blood mononuclear cells (PBMCs) from mice removed from tetracycline for 3 to 18 days. Three founders, #17, #19, and #20 showed high levels of luciferase activity after induction, ranging from 70-900 fold that obtained in extracts of PBMCs from transgene negative mice in the same experiments (Table 1). Founder #11 was leaky and #12 showed no inducible luciferase in PBMCs. It is presumed that variability in inducibility and leakiness of transgenes in different founders is a consequence of the site of integration and/or structure of the integrated transgenes. There was no obvious correlation between levels of luciferase

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expression or leakiness in PBMCs and the copy number of the transgenes. Particularly significant was that when mouse #20 was again given water containing tetracycline for 18 days, after a previous 7 day induction in the absence of tetracycline, luciferase levels dropped essentially to background, demonstrating that transgene induction is reversible (Table 1). Germline transmission of the transgenes from founders #17 and #20, but not #19 was achieved.

**Table 1** Luciferase levels in peripheral blood mononuclear cells of transgenic and control mice

Mouse	Transgene Copy # <sup>a</sup>		Days after removal of tetracycline from drinking water <sup>b</sup>				
	pTet-tTA	pTet-Luc	Day 0	Day 3	Day 7	Day 18	Day 7*
15	-	-	ND	50 (1)	9.4 (1)	ND	ND
21	-	-	11 (1.2)	ND	0	36 (0.5)	ND
13	-	-	ND	ND	60 (1)	69 (1)	ND
17	15	30	ND	1317 (26)	8595 (914)	ND	ND
20	80	120	ND	1137 (23)	7300 (777)	ND	88 (1.3)
11	40	20	943 (100)	ND	3983 (66)	3013 (44)	ND
19	20	40	0 (1)	ND	18250 (304)	ND	ND

<sup>a</sup> The approximate transgene copy number of pTet-tTA and pUHC13-3 (pTet-luc) as estimated from Southern blotting.

<sup>b</sup> Values represent light units (with lysis buffer background subtracted) per  $10^6$  cells measured in cell extracts of PBMCs from mice removed from tetracycline for the indicated number of days. Values in parentheses are the fold increase or decrease in luciferase activities relative to that in cell extracts from a transgene negative mouse in the assay performed the same day.

Day 7\* denotes luciferase activity measured in cell extracts from mice in whose drinking water tetracycline was removed for 7 days and then restored for 18 days.

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To analyze more carefully the inducibility of luciferase in transgenic mice, a variety of tissues and organs of second or third generation transgene positive progeny of founder #17 and #20 (backcrossed to C57Bl/6) were removed from tetracycline for 7 or 8 days and were compared to transgene identical positive progeny maintained on tetracycline. As shown in Figure 4 and Table 2, the progeny of mouse #51 (from Founder #17) showed luciferase activity in all organs examined. Levels of luciferase activity varied substantially between tissues, with expression consistently high in thymus and lung, and low in liver and kidney. Induction ranged from 2-fold in testes to 150-fold in thymus. Luciferase activity ( $10^5$ - $4 \times 10^6$  rLU/mg protein) was also detected in day 17 fetal brain and liver of transgene positive mice conceived in the absence of tetracycline. Additionally, transgene positive mice conceived and maintained from gestation through 3.5 months in the absence of tetracycline continued to express optimal levels of luciferase and appeared normal. Progeny from founder #20 also showed highest levels of inducible luciferase activity in thymus and lung, although inducibility and tissue distribution of luciferase were more restricted than in the progeny of founder #17. Northern blotting demonstrated that tTA mRNA levels were clearly induced in the thymus and lung from progeny of mouse #51 after removal of tetracycline (Figure 3C). Mice removed from tetracycline for up to 6 months appear healthy, indicating that induction of the tTA protein *in vivo* is not toxic or lethal. It was also observed that mRNA hybridizes to a probe specific for the luciferase gene in thymus from induced mice.

**Table 2 Average luciferase activity and fold induction in tissues of transgenic mice<sup>a</sup>**

Tissue	Av. TG Neg.	Av. Unind.	Av. Ind.	Fold Induction
Spleen	107 (9)	684 (8)	33,180 (10)	48
Thymus	220 (9)	16,243 (8)	2,448,580 (10)	151
Lung	138 (9)	1,617 (8)	169,538 (10)	105
Liver	69 (3)	214 (6)	2,022 (8)	9
Kidney	87 (3)	361 (6)	9,440 (8)	26
Heart	0 (3)	5,971 (6)	32,540 (8)	5
Cerebrum	94 (7)	754 (6)	9,836 (8)	13
Cerebellum	91 (7)	904 (6)	67,410 (8)	75
LN	617 (2)	3,892 (4)	74,449 (5)	19
Testes	71 (2)	30,398 (2)	60,911 (3)	2

<sup>a</sup> The average values combine data from the experiments shown in Figure 4. The number of mice in each group is indicated in parentheses. The average fold induction for each tissue is shown. Values represent rLU/mg protein with lysis buffer background (130 rLU to 180 rLU) subtracted.

The autoregulatory system (pTet-tTAK) described here represents a substantial improvement over a constitutive expression strategy (pcDNA-tTAK) in cultured cells, in all likelihood because it prevents toxic effects of the transactivator in the uninduced state and allows for higher levels of transactivator after induction. The constitutive expression strategy is less effective in two regards: a smaller fraction of clones produce any expression at all (18% versus 90%) and induced V(D)J recombinase levels are much lower (by more than 70 fold, averaging over all clones). The kinetics of induction of gene expression with the two systems appears comparable. In preliminary experiments with the autoregulatory system, strong expression of transactivator mRNA is observed 12 hours post induction consistent with the optimal level of protein expression

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observed at 24 hours with the constitutive system (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992)). Stable transfection of pTet-tTak should allow easy derivation of activator cell lines in which a variety of genes can be inducibly expressed by subsequent transient or stable transfection.

5           Previous attempts to create inducible transgenic mice using genes activated by heavy metal ions or aromatic hydrocarbons have been hampered by leakiness, relatively low levels of induction, restricted tissue specificity, and toxicity or carcinogenicity of inducing agents (Jones, S. N. *et al.*, *Nucl. Acids Res.* 19(23):6547-6551 (1991); Goodnow, C. C. *et al.*, *Nature* 342:385-391 (1989);  
10           and reviewed in Yarranton, G. T., *Curr. Opin. Biotech.* 3:506-511 (1992)). The constitutive tetracycline system has been used to create inducible transgenic mice (Furth, P. A., *et al.*, *Proc. Natl. Acad. Sci. USA* 91:9302-9306 (1994)) and avoids some of the difficulties of these earlier approaches. Assuming that equally sensitive luciferase measurement procedures were employed, the autoregulatory  
15           system provides approximately two orders of magnitude more luciferase activity in thymus ( $1.1 \times 10^4$  rLU/mg protein maximum with the constitutive system vs.  $2.5 \times 10^6$  rLU/mg protein with the autoregulatory system) and lung ( $1.5 \times 10^3$  rLU/mg protein maximum with constitutive system vs.  $1.7 \times 10^5$  rLU/mg protein with autoregulatory system). Additional benefits of the autoregulatory system  
20           appear to be a greater induction of luciferase activity in the thymus (150-fold vs. 67-fold), and easily detectable levels of luciferase activity in tissues which show little or no activity in the unmodified system such as lung, kidney and brain. Additionally, since activity in thymus, spleen, and lymph nodes is detected, this system might be especially suited to studies of the immune system. No gross  
25           perturbations of splenic architecture were observed in hematoxylin/eosin stained tissue sections from adult, luciferase expressing, transgenic mice maintained in the absence of tetracycline since conception. As seen with the unmodified system, leakiness varies between tissues, though it is higher in the thymus with the autoregulatory system than with the constitutive system.

By comparison to luciferase protein standards, the luciferase activity that was observed in thymus corresponds to an average of approximately 30 molecules of luciferase per cell. However, it is not known what fraction of cells express luciferase activity or how expression levels vary between expressing cells. Since induction of tTA expression in this system depends upon a low level of leakiness of the tTA transgene, it is expected that inducibility will vary with the transcriptional profiles of individual cell types and stages of differentiation. Therefore, per cell calculations of luciferase protein may under represent the actual levels induced in individual cells.

These results demonstrate that highly inducible and reversible expression from a Tetp-controlled reporter transgene can be obtained using the pTet-tTAk construct, and suggest that mice can develop normally in the presence of tetracycline and these transgenes, and that induction by removal of tetracycline does not lead to any obvious ill-effects on the mice, their ability to breed, or fetal development. Therefore, the potential toxicity of the tTA protein *in vivo* may not be a serious difficulty. Induced mice still express optimal levels of luciferase 3.5 months post tetracycline removal and remain viable at least six months in the absence of tetracycline, suggesting that transgene expression is tolerated and is not downregulated.

The pTet-tTAk system should be able to direct expression of any desired gene or genes in an inducible manner. This expression system should be widely applicable to the study of gene function in transfected cells and *in vivo*, to the creation of disease models for the testing of therapeutic agents, and to efforts to understand the development of mammalian organisms. It will be particularly useful in allowing regulated transgenic expression of genes otherwise too toxic to be tolerated by the organism during development.

## Examples

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature.

### Materials and Methods

#### 5 Details of Plasmid Constructions:

The plasmid pUHC 13-3 (described by Gossen & Bujard, *Proc. Natl. Acad. Sci. USA*:89:5547-5551 (1992)) is 5157 base pairs in size and has three EcoRI restriction sites (at positions 454, 667, 4036) which may be used for a diagnostic restriction digest. The plasmid consists of three main fragments: (1) pBR322-sequences including colE1-origin of replication,  $\beta$ -lactamase-resistance-gene with the  $P_{bla/p3}$  of Tn2661 (HincII-site and PstI-site removed); (2) the regulatory region with hCMV minimal promotor (-53 relative to start site) with heptamerized tet-operators upstream; and (3) the luciferase gene with 3'-flanking region from pSV-2-luc (de Wet, *et al.*, *Mol. Cell. Biol.* 7:725-37 (1987)). See Figure 12A for a map of pUHC 13-3, and Figures 12B-12F for a sequence of pUHC 13-3.

Sequences between the EcoRI and XbaI sites of pUHD15-1 (see Figure 11), Gossen & Bujard, *Proc. Natl. Acad. Sci. USA*:89:5547-5551 (1992), were replaced with a double-stranded oligonucleotide, whose sequence is shown in the top right of figure 7, to generate pCMV-tTAk (step 1). The inserted sequence provides the tTA gene with an optimal context for the initiation of translation, Kozac, M., *Nuc. Acids Res.* 12:857-872 (1984), inserts an amino acid into the tTA protein (alanine at position 2) and provides a unique HindIII site for subsequent cloning steps. This modified tTA gene is herein referred to as tTAk. The HindIII to BamHI fragment of pCMV-tTAk containing the tTAk gene was

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then cloned into the HindIII to BamHI sites of pcDNAI-neo (Invitrogen Corporation) to generate pcDNA-tTAk (step 2 of figure 7).

The plasmid pTet-tTAk places a modified tTA gene called tTAk (abbreviation used herein for the tTA gene with a consensus kozak translation initiation site inserted therein) under control of the Tet promoter of pUHC-13-3 (Gossen & Bujard, *Proc. Natl. Acad. Sci. USA*:89:5547-5551 (1992)). The construct is therefore autoregulatory.

pTet-tTAk was constructed by first constructing a vector with the Tet promoter of pUHC-13-3 in a vector with an SV40 splice and poly-A site and then inserting tTAk between the Tet promoter and the splice/poly A. The SV40 intervening sequence is derived from the small T antigen: Mbo I (0.56mu=4100) to Mbo I (0.44mu=4710). The SV40 poly-A sequence is SV40 from BclI (0.19mu=2770) to EcoRI (0mu=1782), and contains the early polyadenylation sequence and the 3' terminal sequence of the SV40 late region (coordinates given are for SV40). Just upstream of the splice region is 125 bp derived from the 3' end of the bacterial CAT gene (untranslated sequences); these sequences appear to have no harmful effect, and were included only because they provide a convenient restriction site. See Figure 10A for a map of pTet-tTAk and Figures 10B-10G for the sequence of pTet-tTAk.

The starting plasmid for the construction of pTet-tTAk, pSplice-PA, was constructed by inserting the ScaI to EcoRI fragment of pHAV-CAT, Jones, *et al.*, *Nuc. Acids Res.* 19:6547-6551 (1991), into the XbaI site of pBKSII<sup>+</sup> (Stratagene) by ligation of XbaI linkers after Klenow fill in of the EcoRI site. The pSplice-PA plasmid contains the SV40 intervening sequence derived from the small T antigen and the SV40 early polyadenylation sequence, as shown in Figure 8. The XhoI to SalI fragment of pUHC13-3, Gossen & Bujard, *Proc. Natl. Acad. Sci. USA*:89:5547-5551 (1992), which contains seven copies of the *tet* operator upstream of a minimal human cytomegalovirus (hCMV) promoter, was cloned into the unique XhoI site of pSplice-PA to yield pTet-Splice (figure 8, step 1). This *tet* operator-containing promoter is referred to herein as Tetp. pTet-Splice

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contains a number of unique restriction sites for easy insertion of genes of interest between Tetp and the splice/poly A sequences. See Figure 9A for a map of pTet-Splice and Figures 9B-9G for the sequence of pTet-Splice. The HindIII to BamHI (blunted with Klenow) fragment of pCMV-tTAk containing the tTAk gene was then cloned into the HindIII to EcoRV sites of pTet-Splice to yield pTet-tTAk (figure 8, step 2).

The Tetp-controlled mouse RAG-1 expression construct used in these experiments was constructed by inserting the coding region of pR1A/C as a BamHI (blunted with Klenow) to XbaI fragment into the EcoRV to SpeI sites of pTet-Splice to yield pTet-R1A/C. However, the nucleic acid molecule encoding RAG-1 can be synthesized chemically using known methods in the art or can be isolated from any other source. For the complete sequence of RAG-1, see Schatz, D. G. *et al.*, *Cell* 59:1035-1048 (1989). The RAG-1 coding region of pR1A/C has been altered, as compared to full length mouse RAG-1, by deletion of amino acids 2-89 and amino acids 1009-1040, the addition of six histidines immediately following the second codon, the insertion of alanine and serine codons immediately after the histidines to introduce a site for NheI, and the addition of a consensus translation initiation context surrounding the AUG start codon. This deletion mutant of RAG-1 has significantly greater V(D)J recombinase activity than full length mouse RAG-1.

The tetracycline-controlled mouse RAG-2 expression construct used here was constructed by inserting the XhoI to XbaI fragment of pR2A-CDM8 into the SalI to SpeI sites of pTet-Splice to yield pTet-R2A. However, the nucleic acid molecule encoding RAG-2 can be synthesized chemically using known methods in the art or can be isolated from any other available source. For the complete sequence of RAG-2, see U.S.P.N. 5,159,066, issued October 27, 1992, or Oettinger, M. A. *et al.*, *Science* 248:1517-1523 (1990). The RAG-2 coding region of pR2A-CDM8 has been altered, relative to full length mouse RAG-2, by the C-terminal deletion of amino acids 492-527, the addition of a consensus translation initiation context surrounding the AUG start codon, and the insertion

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of an alanine codon at position 2. This RAG-2 mutant has somewhat higher V(D)J recombinase activity than full length mouse RAG-2.

***Example 1: Construction of plasmids***

The EcoRI-BamHI fragment of pUHD15-1 (Gossen, M. & Bujard, H.,  
5 *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992)) containing the tTA open  
reading frame (ORF) was modified at its 5' end by addition of a 30 bp  
oligonucleotide (SEQ ID NO:\_\_\_) to provide an optimal context for translational  
initiation (Kozak, M., *Nucl. Acids Res.* 12:857-872 (1984)) and a unique HindIII  
10 site for subsequent cloning. The added nucleotides are those shown at the top  
right, Figure 7. The *number* of added nucleotides depends on whether one counts  
the nucleotides of the EcoRI and XbaI enzyme sites. For simplicity, the modified  
5' end of the ORF is referred to herein as a 30 bp oligo. The modified tTA gene  
is hereafter referred to as tTAk. The HindIII-BamHI tTAk fragment was cloned  
15 into the HindIII-BamHI sites of pcDNAI-neo (Invitrogen Corporation) to yield  
pcDNA-tTAk. In pcDNA-tTAk, the tTAk gene is under the transcriptional  
control of the enhancer and promoter sequences of the immediate early gene of  
human cytomegalovirus (hCMV). The plasmid pSplice-PA was constructed by  
inserting the SV40 small T antigen intervening sequence and the SV40 early  
polyadenylation sequence from pHAV-CAT (Jones, S. N. *et al.*, *Nucl. Acids Res.*  
20 19:6547-6551 (1991)), into pBKSII<sup>+</sup> (Stratagene). The XhoI-SalI fragment of  
pUHC13-3 (Gossen, M. & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551  
(1992)), containing seven copies of the *tet* operator upstream of a minimal  
promoter (hereafter referred to as Tetp), was cloned upstream of the splice/polyA  
sequences of pSplice-PA to yield pTet-Splice. The tTAk gene was cloned into  
25 pTet-Splice to yield pTet-tTAk, placing the start site of transcription 143 bases  
upstream of the tTAk AUG (Figure 1).

The Tetp-controlled mouse RAG-1 expression construct (pTet-R1A/C)  
was constructed by inserting the coding region of pR1A/C into pTet-Splice. The

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RAG-1 coding region of pR1A/C has been altered, as compared to full length mouse RAG-1, by small N and C terminal deletions which result in at least a two-fold increase in V(D)J recombinase activity, *i.e.* two fold increase in VDJ with R1A/C over R1A and R2A over R2. The Tetp-controlled mouse RAG-2 expression construct (pTet-R2A) was constructed by inserting the coding region of pR2A-CDM8 into pTet-Splice. This RAG-2 coding region is altered relative to full length mouse RAG-2 by a small C-terminal deletion which results in a small increase in V(D)J recombinase activity. pTet-R1 and pTet-R2 consist of the complete RAG-1 and RAG-2 ORFs, respectively, inserted into pTet-splice.

#### ***Example 2: Cell culture and derivation of transfected cell lines***

Stable transfectants of pcDNA-tTAK were generated by calcium phosphate/glycerol shock transfection of 10 µg of linearized plasmid into 0.5 x 10<sup>6</sup> NIH 3T3 fibroblast cells as described (Schatz, D. G. *et al.*, *Cell* 59:1035-1048 (1989)) and 48 hours after transfection plating cells in 0.75 mg/ml G418 plus 0.5 µg/ml tetracycline. Single colonies picked after 12 days were expanded in 0.5 mg/ml G418, 0.5 µg/ml tetracycline.

Stable transfectants of pTet-tTAK alone or pTet-tTAK plus pTet-R1A/C plus pTet-R2A, were generated by transfecting 10 µg of each linearized plasmid with 1 µg of linearized pSV2-His, followed by selection in media containing L-histidinol but lacking histidine as described previously (Schatz, D. G. *et al.*, *Cell* 59:1035-1048 (1989)). Transfected cells were maintained in the presence of 0.5 µg/ml tetracycline, beginning at the time of transfection.

#### ***Example 3: Assay for V(D)J recombinase activity***

V(D)J recombinase activity was measured using the extrachromosomal reporter plasmid pD243 (a signal joint deletion substrate) as described by others (Lewis, S. M. & Hesse, J. E., *EMBO J.* 10(12):3631-3639 (1991)). Briefly,

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NIH3T3 fibroblast cell lines were transfected with 10 µg of pD243, and where indicated 6 µg of pTet-R1A/C and 4.8 µg of pTet-R2A, by the calcium phosphate/glycerol shock transfection method. Tetracycline was omitted from the culture medium after the transfection in the samples indicated "tet". In other cases ("tet<sup>+</sup>"), cells were maintained in media containing 0.5 µg/ml tetracycline. Extrachromosomal plasmid molecules were harvested by rapid alkaline lysis of the cells 48 hours after transfection, and a small aliquot of the isolated DNA was electroporated into MC1061 bacteria. The electroporated bacteria were spread on LB agar plates containing 100 µg/ml ampicillin (A) and on plates containing 11 µg/ml chloramphenicol and 100 µg/ml ampicillin (CA). After sixteen hours of growth at 37°C the percent recombination, R<sub>n</sub>, was calculated as the total number of CA resistant colonies divided by the total number of A resistant colonies, multiplied by 100. Greater than 99% of plasmids harvested from NIH3T3 fibroblasts 48 hours after transfection have replicated at least once (as indicated by their resistance to digestion by DpnI), demonstrating that essentially all of the harvested plasmid molecules have entered the nucleus of transfected cells and are therefore assumed to have been accessible for recombination (Lieber, M. R. *et al.*, *Genes and Devel.* 1:751-761 (1987)).

#### ***Example 4: RNA blot analysis***

Electrophoresis of total cell RNA in 1-1.2% agarose/formaldehyde gels was followed by blotting to nylon membranes (Zetabind, CUNO or Genescreen Plus, NEN) and subsequent hybridization with DNA probes prepared using a random hexamer labelling kit (Boehringer Mannheim). Probes detecting RAG-1 and RAG-2 mRNA were prepared from fragments of the RAG-1 or RAG-2 coding regions (Schatz, D. G. *et al.*, *Cell* 59:1035-1048 (1989); Oettinger, M. A. *et al.*, *Science* 248:1517-1523 (1990)), respectively. The probe for actin has been described previously (Schatz, D. G. *et al.*, *Cell* 59:1035-1048 (1989)).

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**Example 5: Western blot analysis**

Protein from  $1.5 \times 10^7$  cells per lane was subjected to SDS-PAGE on an 8% polyacrylamide gel and electroblotted to a 0.2 micron PVDF membrane (BIO-RAD Laboratories). Membranes were blocked at room temperature (RT) overnight in a solution of 1%BSA, 0.5% gelatin, in TTBS (Tris buffered saline + 0.1% Tween-20), washed 2 X 5 minutes in TTBS and probed overnight with a monoclonal anti-*tet* R antibody (9F10)-containing hybridoma supernatant (S. Freundlieb and H. Bujard, Heidelberg, Germany), diluted 1:4 in 1%BSA in TTBS. The blots were washed 4 x 10 minutes in TTBS and tTA protein was detected by incubation for 40 minutes with goat anti-mouse antibody (1:10,000 in TTBS) (Amersham), washing in TTBS 4 x 10 minutes and TBS 2 x 10 minutes, and subsequent developing with an ECL western blotting kit (Amersham).

**Example 6: Transgenic mice and assays for luciferase**

Mice doubly transgenic for pTet-tTAk (XhoI to NotI fragment) and pUHC13-3 (XhoI to AseI fragment) were created by co-microinjection of gel purified DNA (in the presence of 0.5  $\mu$ g/ml tetracycline) into fertilized F1 (C57BL/6 x C3H) eggs, which were then implanted into the uterus of pseudopregnant females. Pregnant females were provided with water containing 100  $\mu$ g/ml tetracycline and 5% sucrose. Progeny were screened by probing Southern blots of tail DNA with tTA (761 bp XbaI-SalI) or luciferase (1365 bp HindIII-EcoRV) fragments labeled with  $\alpha$ - $^{32}$ P-dCTP as above. Transgene copy number was estimated by comparison of the Southern blot signal to those obtained from dilutions of plasmid DNA fragments.

Luciferase activity in tissues of transgenic mice was measured using an assay system according to the manufacturers instructions (#E1500, Promega Corporation). Peripheral blood mononuclear cells (PBMCs) ( $0.1$ - $1.0 \times 10^6$  cells)

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5 were lysed in 50  $\mu$ l of lysis buffer for 15 minutes at room temperature, and after pelleting the insoluble material for 5 seconds at 14,000 rpm, 20  $\mu$ l of the supernatant was mixed with 100  $\mu$ l of luciferin reagent and the light produced in 10 seconds was measured in a luminometer (Berthold, Lumat LB9501, Germany). The number of cell equivalents of lysate in the assay was used to  
10 normalize luciferase activity between samples. Other tissues, harvested and quick frozen in liquid nitrogen, were ground to a powder with a cold mortar and pestle, placed in 100-200  $\mu$ l luciferase lysis buffer, and incubated at RT for 15 minutes. Cell debris was pelleted for 10 seconds at 14,000 rpm and supernatant was stored at -70°C until analysis. 20  $\mu$ l supernatant was used in luciferase assays. For  
15 normalization of luciferase activity between tissue lysates, total protein concentration in lysates was determined using a Bradford protein assay (Bio-Rad Laboratories). Samples were assayed within the linear range of the assay and only approximately 2-fold variation was observed as lysates were diluted. Firefly luciferase protein standard (Sigma) added to extracts from a variety of tissues from wild type mice showed no variation in activity.

20 While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims. All patents and publications mentioned herein are incorporated by reference in their entirety.

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*What Is Claimed Is:*

1. A composition of matter comprising a polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence.

2. A composition of matter as claimed in claim 1, wherein the open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is modified at its 5' end to provide an optimal context for translational initiation.

3. A composition of matter as claimed in claim 2, wherein the 5' end of the open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is further modified to provide a unique restriction site.

4. A composition of matter as claimed in claim 3, wherein the unique restriction site is *HindIII*.

5. A composition of matter as claimed in claim 4, wherein the open reading frame of the polynucleotide molecule encoding the tetracycline transactivator fusion protein is modified at its 5' end to encode an oligonucleotide identified as SEQ ID NO. -----.

6. A composition of matter as claimed in claim 1 which is DNA.

7. A composition of matter comprising a cloning vector containing the polynucleotide molecule of claim 1.

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8. A composition of matter comprising a eucaryotic cell transfected with the polynucleotide molecule of claim 1.

9. A composition of matter as claimed in claim 8, wherein said eucaryotic cell is further transfected with a polynucleotide molecule encoding a heterologous protein operably linked to an inducible minimal promoter, which contains at least one *tet* operator sequence.

10. A composition of matter as claimed in claim 9, wherein at least one of the polynucleotide molecules is operably linked to a minimal promoter and seven *tet* operator sequences.

11. A method to decrease or shut off expression of a heterologous protein comprising

(a) transforming a eucaryotic cell with

(i) a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence;

(ii) a second polynucleotide molecule encoding the heterologous protein, said protein being operably linked to an inducible minimal promoter, and said promoter containing at least one *tet* operator sequence; and

(b) cultivating the eucaryotic cell in a medium comprising tetracycline or a tetracycline analogue.

12. A method as claimed in claim 11, wherein the second polynucleotide molecule is operably linked to a minimal promoter and seven *tet* operator sequences.

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13. A method to activate or enhance the expression of a heterologous protein comprising

(a) transforming a eucaryotic cell with

5 (i) a first polynucleotide molecule encoding tetracycline transactivator fusion protein, said protein comprising a prokaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible promoter, which promoter contains at least one *tet* operator sequence;

10 (ii) a second polynucleotide molecule encoding the heterologous protein, said protein being operably linked to an inducible minimal promoter, and said promoter containing at least one *tet* operator sequence; and

(b) cultivating the eucaryotic cell in a medium lacking tetracycline or a tetracycline analogue.

14. A composition of matter as claimed in claim 8 containing  
15 tetracycline in an amount sufficient to suppress binding of tetracycline transactivator fusion protein to said inducible minimal promoter.

15. A composition of matter as claimed in claim 9, wherein the  
20 polynucleotide molecule encoding a tetracycline transactivator fusion protein is expressed in an amount sufficient to drive expression of the polynucleotide molecule, encoding the heterologous protein, in the absence of tetracycline.

16. A composition of matter as claimed in claim 9 wherein  
tetracycline transactivator fusion protein is present in an amount sufficient to drive expression of the heterologous protein.

17. A composition of matter consisting essentially of the plasmid  
25 pTet-Splice.

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18. A composition of matter consisting essentially of the plasmid pTet-tTAK.

19. A kit comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a procaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence; and a second container means contains a second polynucleotide molecule encoding said inducible minimal promoter, which promoter contains at least one *tet* operator sequence, which *tet* operator sequence is strategically positioned for being operably linked to a heterologous polynucleotide sequence encoding a polypeptide.

20. A kit comprising a carrier means having in close confinement therein at least two container means, wherein a first container means contains a eucaryotic cell transfected with a first polynucleotide molecule encoding a tetracycline transactivator fusion protein, said protein comprising a procaryotic *tet* repressor and a eucaryotic transcriptional activator protein, and said polynucleotide molecule being operably linked to an inducible minimal promoter, which promoter contains at least one *tet* operator sequence; and a second container means contains a second polynucleotide molecule comprising an inducible minimal promoter, which promoter contains at least one *tet* operator sequence, which *tet* operator sequence is strategically positioned for being operably linked to a heterologous polynucleotide sequence encoding a heterologous polypeptide.

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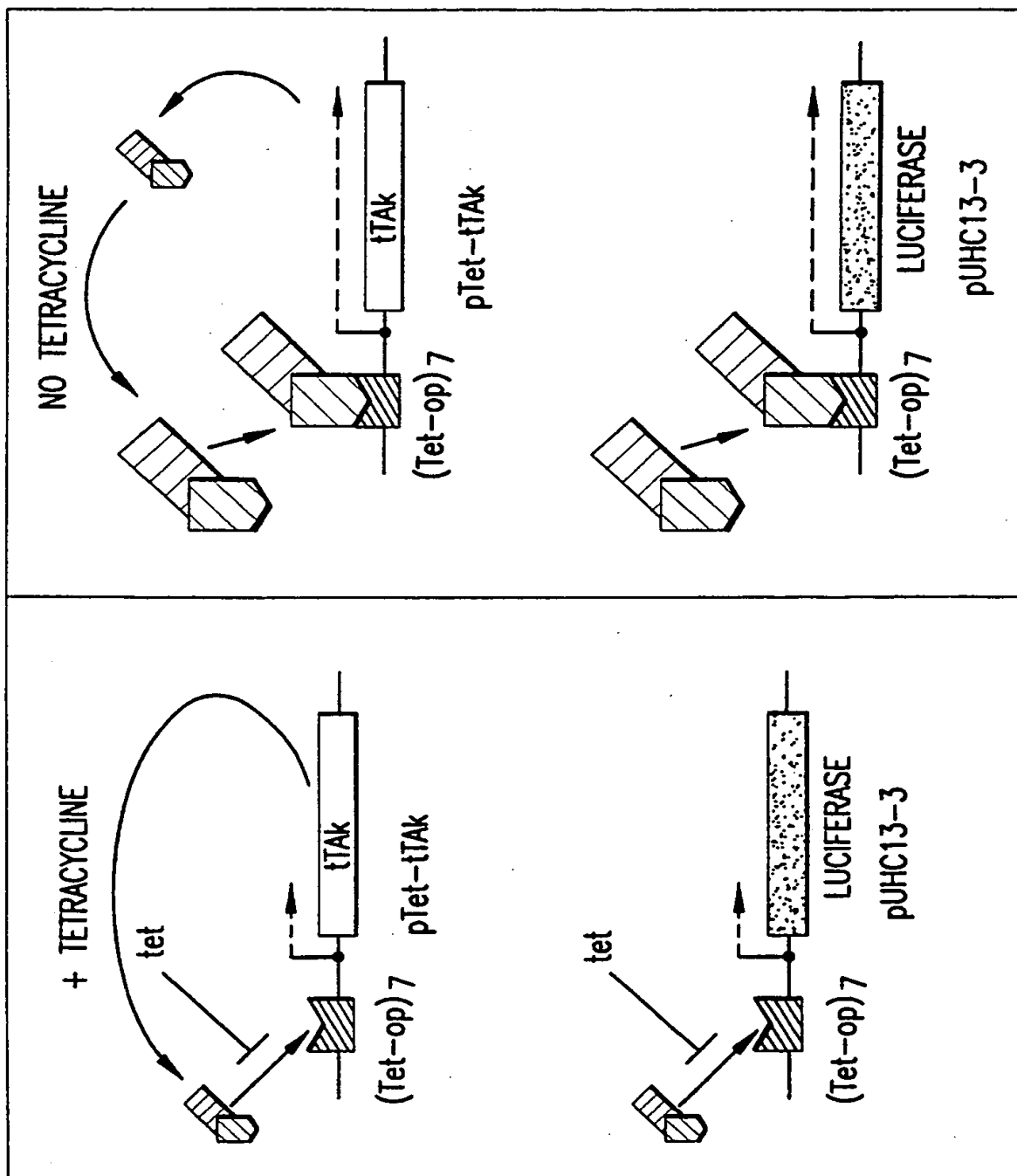


FIG.1

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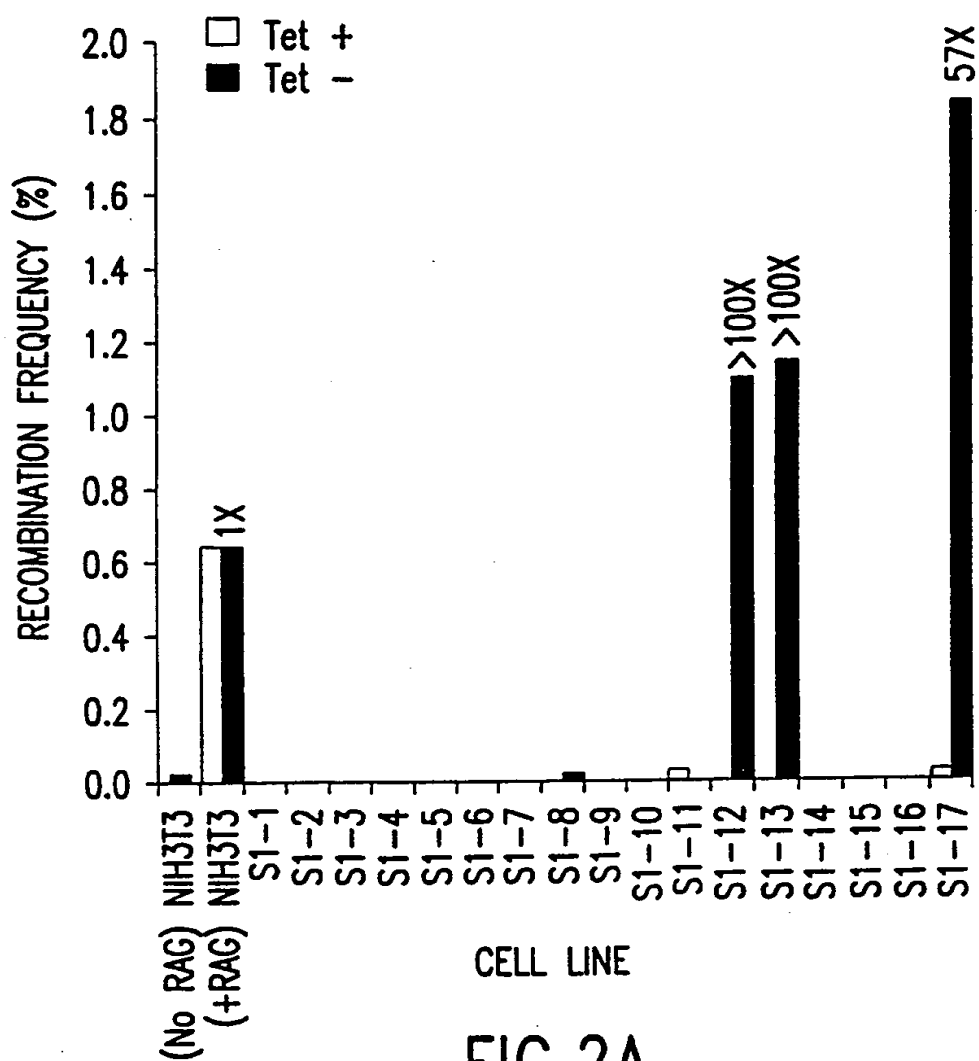


FIG.2A

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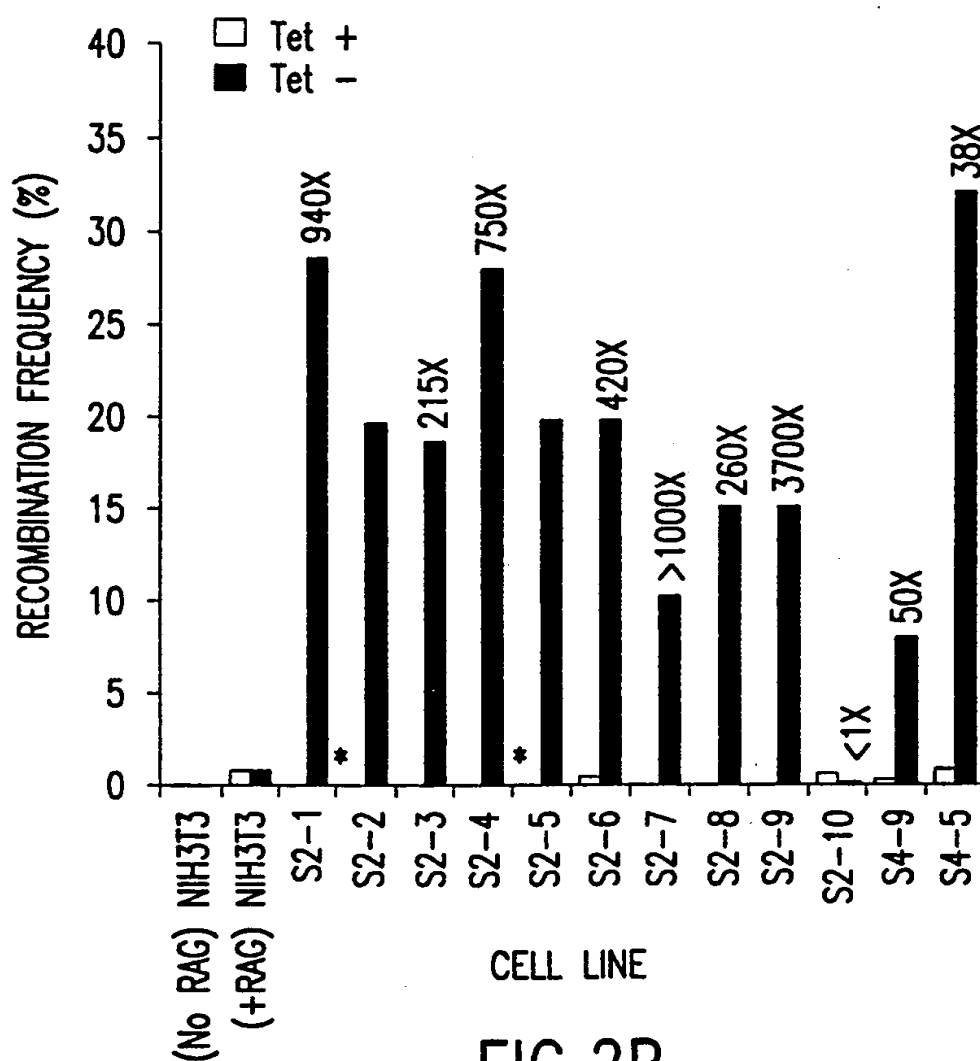


FIG.2B

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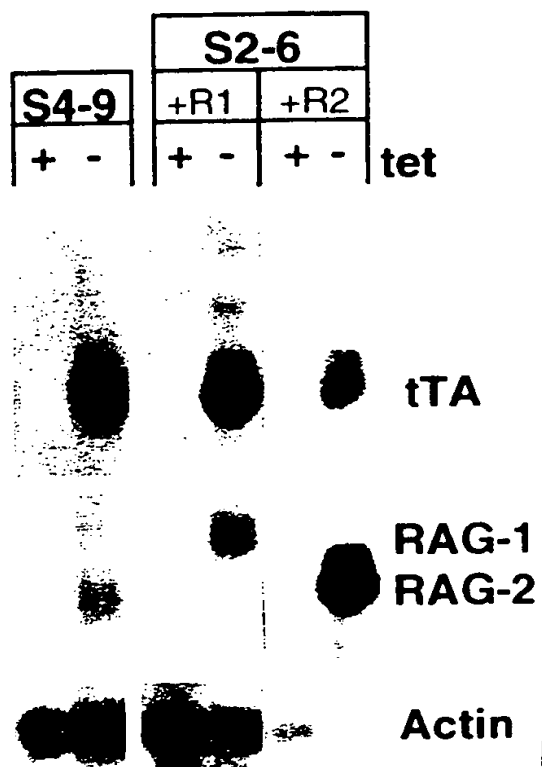


FIG. 3A

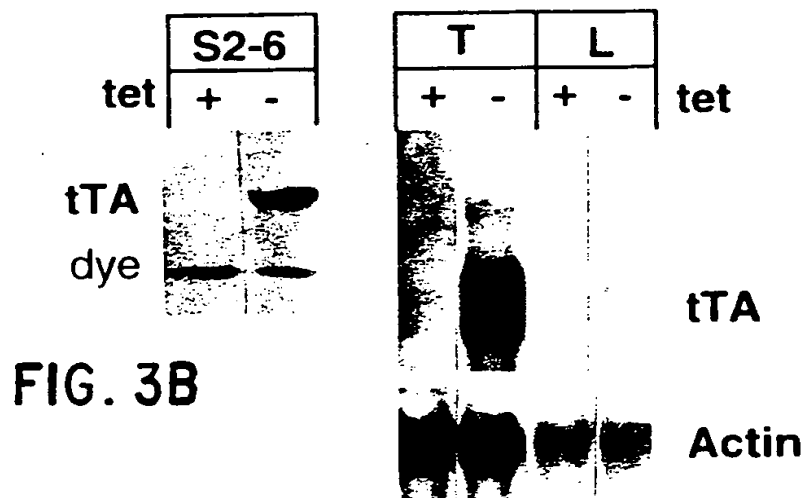


FIG. 3B

FIG. 3C

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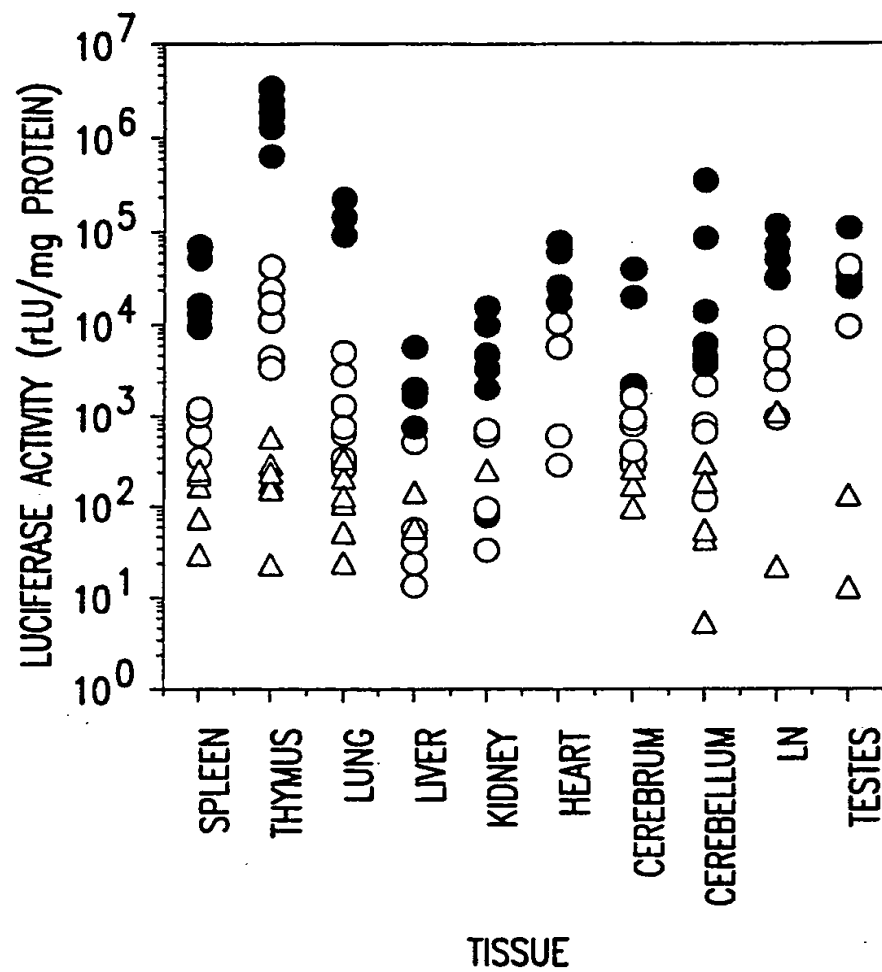


FIG. 4

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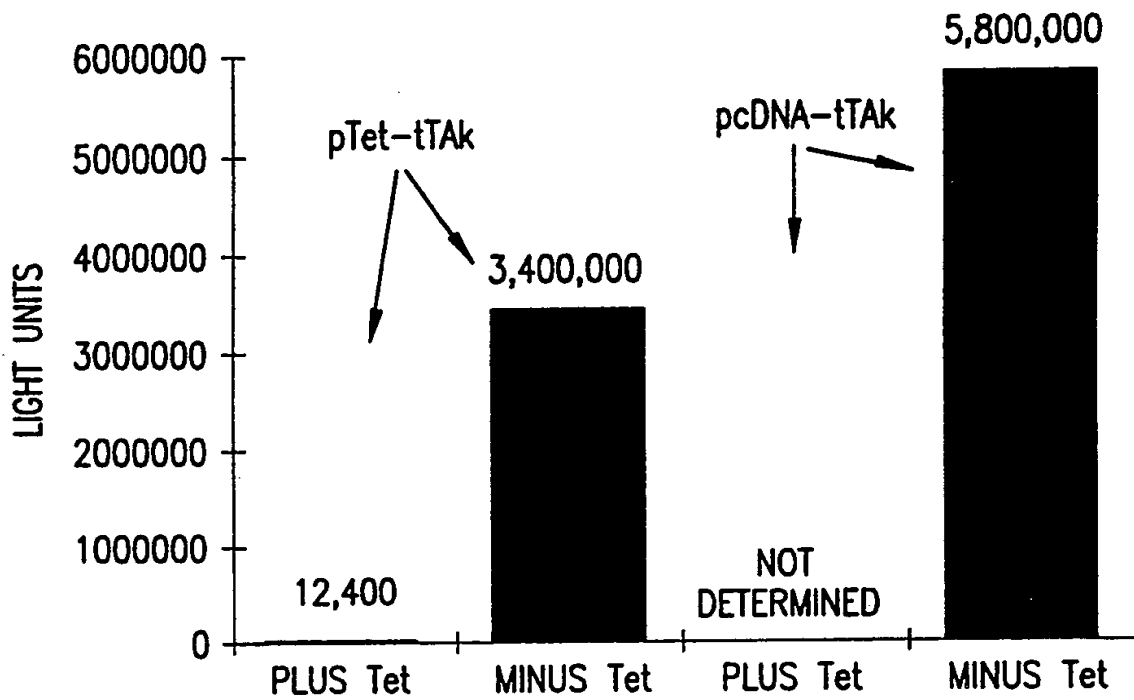
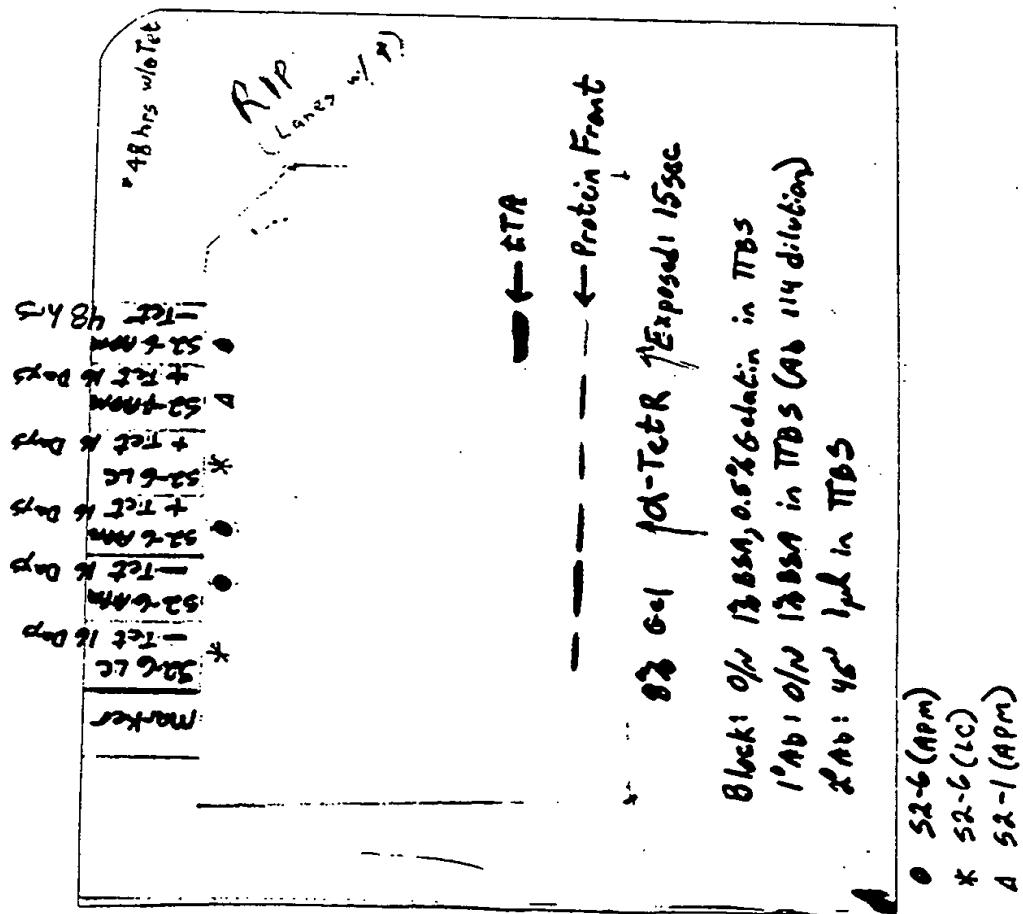
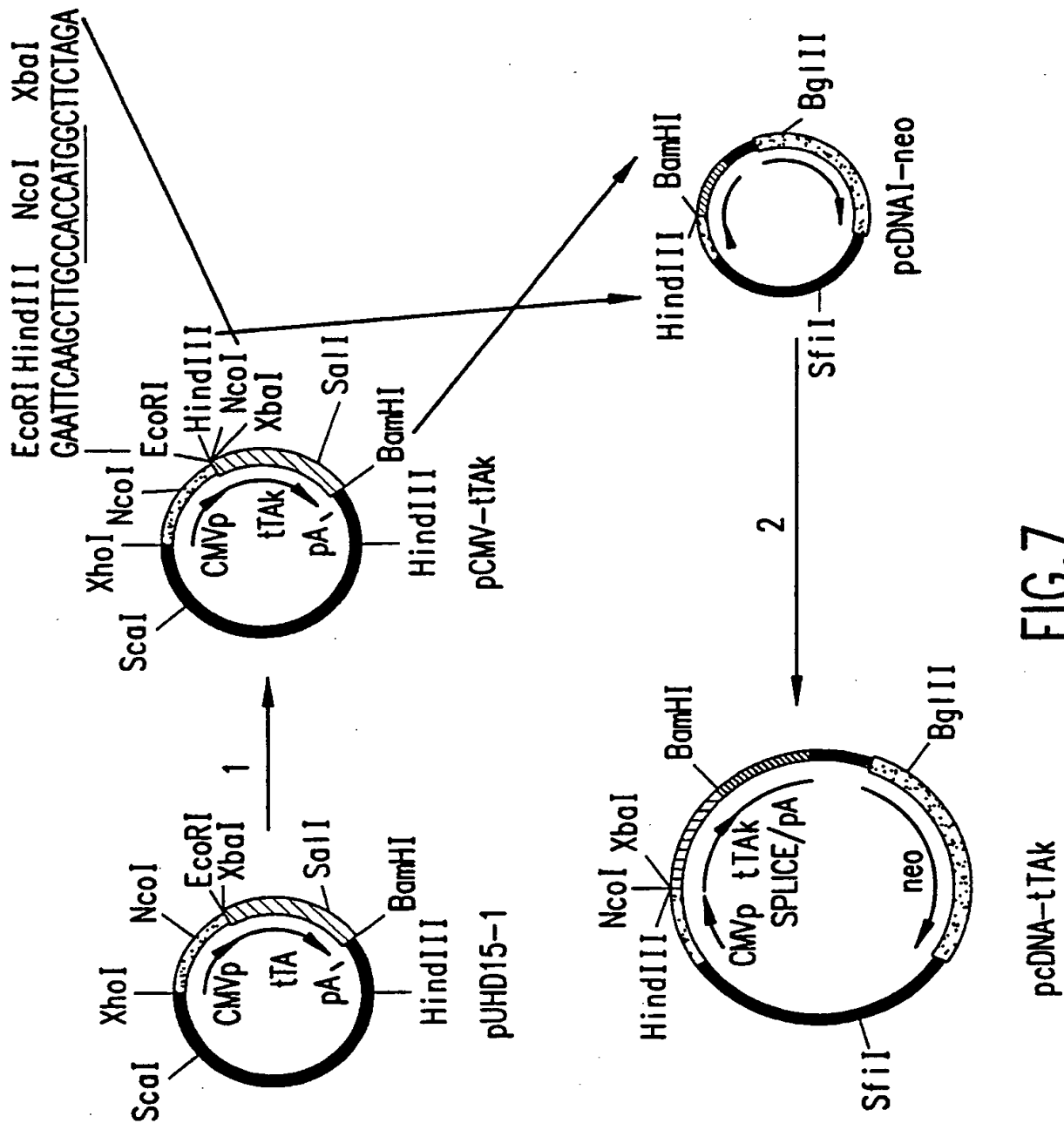


FIG.5

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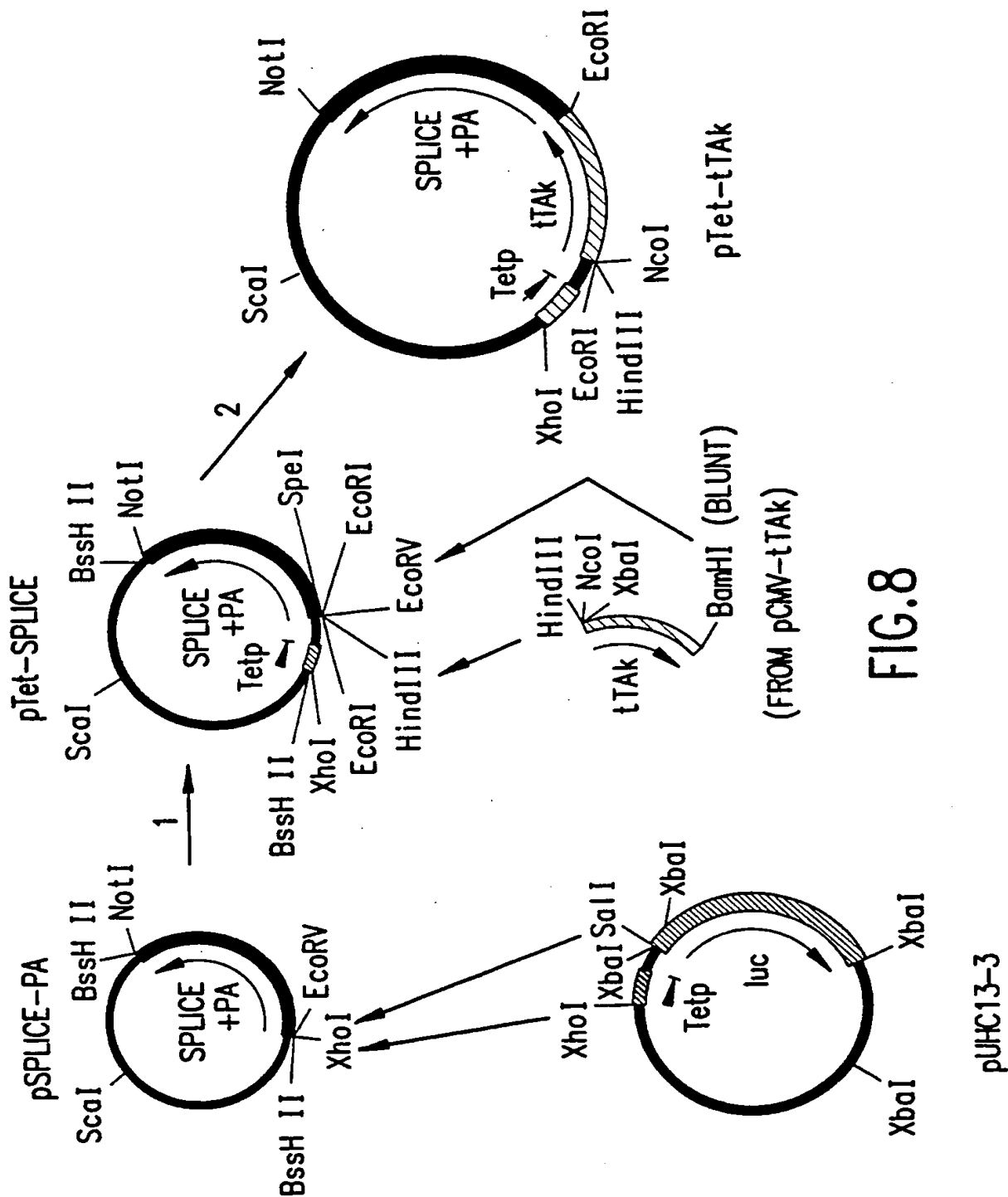


FIG.8

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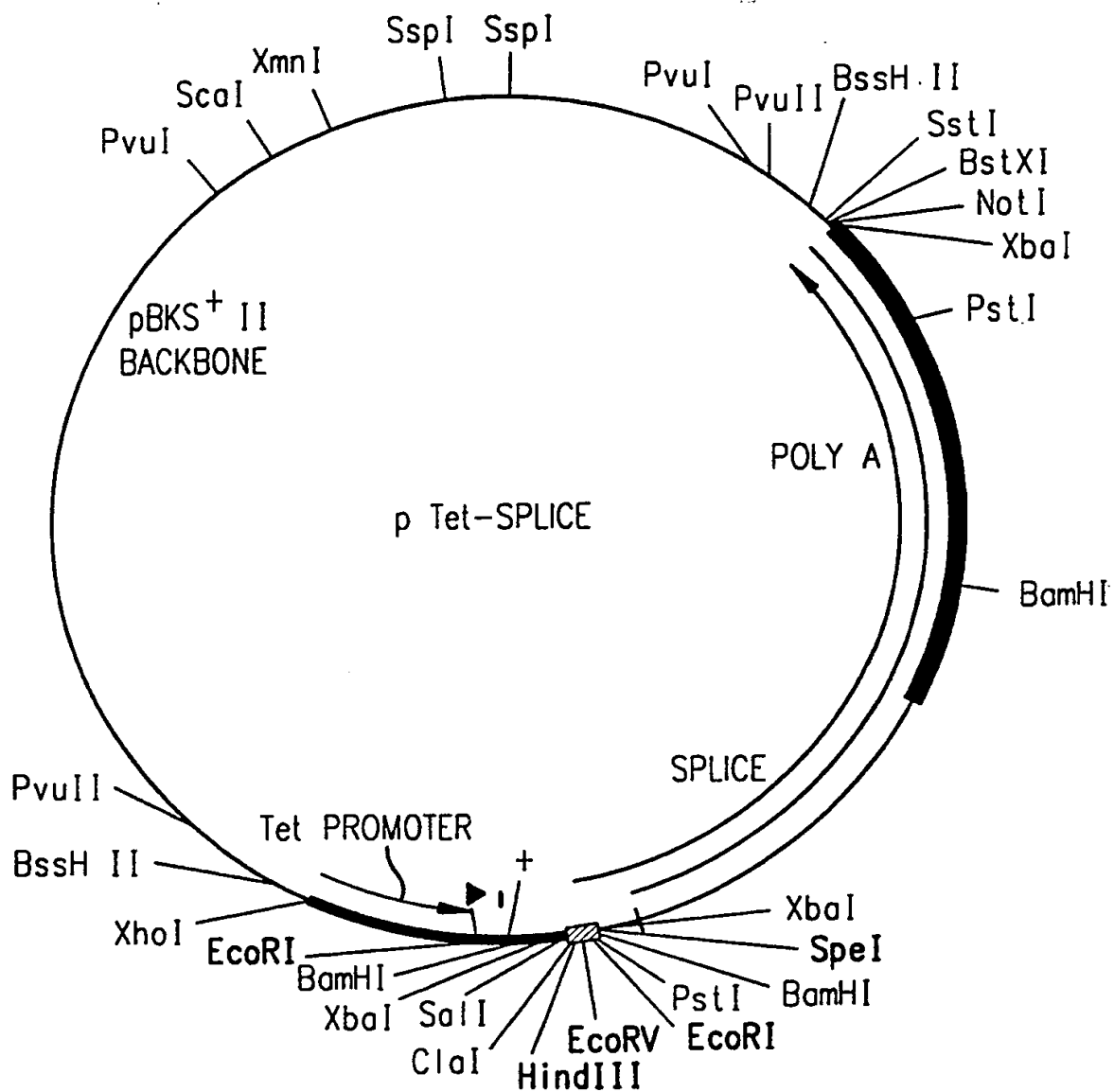


FIG.9A

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SspI

1 CTAATGTAGCGTTAATATTTTGTAAATTCGGTTAAATTTTGTAAATCAGCTCATTTTTTAACCAATAGGCCG  
81 AAATCGGCAAAATCCCTTATAAATCAAAAGAAATAGACGAGATAGGTTGAGTTGTTCCAGTTTGGAAACAAGAGTCCA  
161 CTAITAAAGAACGTGGACTCCAACGTCAAAGGCGAANAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACC  
241 CTAATCAAGTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCTAAAGGGAGCCCCGATTTAGAGCTTGAC  
321 GGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGAAGCGAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCG  
401 GTCAGCTGCGCGTAACCAACACACCGCCGCTTAATGCGCGCTACAGGGCGGTCCTCCATTCGCCATTCAGGCTGCG  
PvuII PvuII  
481 CAACTGTTGGGAAGGCGATCGGTGCGGGCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGATGTGCTGCAAGGCGAT  
BssHI  
561 TAAGTTGGGTAACGCCAGGTTTTCCAGTCACGAGTTGTAAACGACGGCCAGTGAGCGCGGTAATACGACTCACTA  
SstI BstXI NotI XbaI  
641 TAGGGCGAATTGGAGCTCCACCGCGGTGGCGCGCTCTAGAGCAATTCCTTTGCCIAATTTAAATGAGGACTTAACCTG  
721 TGGAAATATTTGATGTGGGAAGCTGTACTGTTAAAACGTAGGTTATTGGGGTAAGTCTATGTTAAACTTGCATTTCAG  
801 GGACACAAAAAAGTCAATGAAATGGTGCTGGAAAAACCCATTCAAGGGTCAAAATTTTTCATTTTTTGCTGTTGGTGGGAA  
PstI  
881 CCTTGGAGCTGCAGGGTGTGTAGCAAACTACAGGACCAAAATATCCTGCTCAAACTGTAAACCCCAAAAAATGCTACAGT

FIG. 9B

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961 TGACAGTCAGCAGATGAACACTGACCACAAGGCTGTTTTGGATAAGGATAATGCTTATCCAGTGGAGTGCTGGGTTCCTG  
1041 ATCCAAGTAAAAATGAAAACACTAGATATTTTGGAACTACACAGGTGGGGAATAATGTGCTCCTGTTTTGCACATTACT  
1121 AACACAGCAACCACAGTGCTTCTTGATGAGCAGGGTGTTGGGCCCTTGTCGCAAGCTGACAGCTTGATGTTTCGCTGT  
1201 TGACATTTGTGGGCTGTTACCAACACTTCTGGAACACAGCAGTGGGAAGGACTTCCCAGATAATTTAAAAATTACCCCTTA  
1281 GAAAGCGTCTGTGAAAAACCCCTACCCAATTCCTTTTGTAAAGTGACCTAATTAAACAGGAGGACACAGAGGGTGGAT  
1361 GGGCAGCCTATGATTGGAA TGCTCTCAAGTAGAGGAGTTAGGGTTTATGAGGACACAGAGGAGCTTCCTGGGGATCC  
1441 AGACATGATAAGATACATTGATGAGTTGGACAAACCACAACTAGAAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTT  
1521 GTGATGCTATTGCTTTATTTGTAACCATTAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCAATTTATGTTT

BamHI

FIG. 9C

SUBSTITUTE SHEET (RULE 26)

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1601	CAGGTTCAAGGGGAGGTGTGGAGGTTTTTAAAGCAAGTAAACCTCTACAAATGTGGTATGGCTGATTATGATCTTTG
1681	TGAAGGAACCTTACTTCGTGGTGTGACATAATTGGACAACTACCTACAGAGATTTAAAGCTCTAAGCTAAATATAAAA
1761	TTTTAAGTGATAATGTGTTAACTACTGATTCATAATTGTTGTGTAATTTAGATTCCAACCTATGGAACTGATGAATG
1841	GGAGCAGTGGTGAATGCCCTTAAATGAGGAAACCTGTTTTGCTCAGAGAAATGCCATCTAGTGATGATGAGGCTACTG
1921	CTGACTCTCAACATTCTACTCCTCCAAAAGAGAGAAAGGTAGAAGACCCCAAGGACTTTCCTTCAGAAATTGCTAAGT
2001	TTTTTGAGTCATGCTGTGTTTAGTAATAGAACTCTTGCTTGCTTTTGCTATTTACACCACAAAGGAAAAAGCTGCACGTCT
2081	ATACAAGAAATTATGGAAAAATATCTGTAACCTTTATAAGTAGGCATAACAGTTATAATCATAACATACTGTTTTTTC
2161	TTACTCCACACAGGCATAGAGTGTCTGCTATTATAACTATGCTCAAAAAATTGGTACCTTTAGCTTTTAAATTGTAAA

FIG. 9D

SUBSTITUTE SHEET (RULE 26)

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2241 GGGGTTAATAAGGAATATTTGATGTATAGTGCCTTGACTAGAGATCGCCTCGGCGGAATTTCTGCCATTATCGCGCTTA  
 2321 TTATCACTTATTCAGGCGTAGCACAGGCGTTTAAGGGCACCAATAACTGCCCTTAAAAAATTAGGCCCGGCCCTGCCAC  
 2401 TCATCGCAGTGTCTAGAACTAGTGGATCCCCCGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGA  
 2481 CTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGGGGCCGGAGGCTGGATCGGTCCGGTGTCTTCTATGGAGGTCA  
 2561 AACAGCGTGGATGGCGTCTCCAGGCGATCTGACGGTTCACCTAACGAGCTCTGCTTATATAGGCCCTCCACCGTACACG  
 2641 CCTACTCGACCCGGGTACCGAGCTCGACTTTTCTCTATCACTGATAGGGAGTGGTAACTCGACTTTCACTTT  
 2721 CTCTATCACTGATAGGGAGTGGTAACTCGACTTTTCTCTATCACTGATAGGGAGTGGTAACTCGACTTTCAC  
 2801 TTTTCTCACTGATAGGGAGTGGTAACTCGACTTTTCTCTATCACTGATAGGGAGTGGTAACTCGACTT  
 2881 TCACCTTTTCTCTATCACTGATAGGGAGTGGTAACTCGACTTTTCTCTATCACTGATAGGGAGTGGTAACTCG

XbaI SpeI BamHI EcoRI PstI EcoRI HindIII SalI  
 EcoRV ClaI

XbaI BamHI EcoRI  
 + ← start site of trxn (?)  
 ← TATA BOX

XhoI

FIG. 9E

SUBSTITUTE SHEET (RULE 26)

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## BssH II

2961 AGGGGGGGCCCGGTACCCAGCTTTTGTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTT  
3041 TCCGTGTGAAATTGTTATCCGCTCACAAATCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCT  
PvuII

3121 AATGAGTGAGCTAACTCACATTAAATTGCGTTGCGTCACTGCCCGCTTCCAGTGGGAAACCTGTGTCGCCAGCTGCAT  
3201 TAAATGAATCGGCCAACGCGGGGAGAGCGGTTTGGGTATTTGGCGCTCTCCGCTTCCCTCGCTCACTGACTCGCTGCG  
3281 CTCGGTCGTTCCGGCTCGGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAAACGGTTATCCACAGAAATCAGGGGATAACG  
3361 CAGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGCCAGGAACCGTAAAAAAGGCCGCTTGTGGCGTTTTTCCATAGG  
3441 CTCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCA  
3521 GGGCTTCCCCCTGGAAGCTCCCTCGTGGCTCTCCTGTTCCGACCTGCCGCTTACCGGATACCTGICCGCTTTCTCC  
3601 CTTCCGGAAGCGTGGCGCTTCTCATAGCTCAGCTGAGGTATCTCAGTTCCGTTGAGTGTGCTCGCTCCAAAGCTGGGC  
3681 TGTGTGACGAAACCCCGCTCAGCCGACCGCTGCCCTTATCCGGTAACTATCGCTTGAAGTCAAGTCCAAACCGGTAAAGACA  
3761 CGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGA  
3841 AGTGGTGGCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGGCTCTGCTGAAGCCAGTTACCTTCGGAAAA  
3921 AGAGTTGGTAGCTCTTGATCCGGCAACAAACCCGCTGGTAGCGGTGGTTTTTGTGTTGCAAGCAGCAGATTACGCG  
4001 CAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTCTACGGGCTGACGCTCAGTGGAAACGAAACTCACGTTAAG  
4081 GGA TTTTGGTCATGAGATTATCAAAAGGATCTTCACTAGATCCCTTTAAATTAAAAATGAAGTTTAAATCAATCTAA  
4161 AGTATATAGATAACTTTGGTCTGACAGTTACCAATGCTTAAATCAGTGAAGGCACCTATCTCAGCGATCTGCTATTTCG  
4241 TTCAATCCATAGTTGCCGTGACTCCCCGTGCTAGATAACTACGATACGGGAGGCTTACCATCTGGCCCCAGTGTGCAA  
4321 TGATACCGGAGACCCACGCTCACCGGCTCCAGATTATCAGCAATAAACCCAGCCAGCCGGAAGGCCGAGCGCAGAAGT  
4401 GGTCTGCAACTTTATCCGCTCCATCCAGTCTATTAAATTGTTGCCGGAAGCTAGAGTAAGTAGTTCCGCCAGTTAATAG  
4481 TTTGCGCAACGTTGTGGCATTGCTACAGGCATCGTGGTGTACCGCTCGTCTTGGTATGGCTTCATTCAGCTCCGGTT  
PvuI

4561 CCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAGCGTTAGCTCCTTCGGTCTCCGATCGTTGTGTC  
4641 AGAAGTAAGTTGGCGCAGTGTATCACTCATGTTATGGCAGCACTGCATAATTCTCTTACTGTGATGCCATCCGTAAG

FIG. 9F

SUBSTITUTE SHEET (RULE 26)

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4721 ATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGATGCGGCGACCGAGTTGCTCTTGCCCGG  
ScaI  
4801 CGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGTCTCATCATTTGGAAAACGTTCTTCGGGGCGAAAA  
4881 CTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCCAACTGATCTTCAGCATCTTTTAC  
4961 TTTCAACAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCCAAAATGCCGCCAAAAAAGGGAATAAGGGCGACACGGAAATGTT  
SspI  
5041 GAATACTCATCTCTTCCTTTTTCATAATTATTGAAGCATTATCAGGGTTATTGTCTCATGAGCGGATACATAATTTGAA  
5121 TGTATTTAGAAAAATAAACAAATAGGGGTTCGGCGCACATTTCCCCGAAAAGTGCCAC

FIG. 9G

SUBSTITUTE SHEET (RULE 26)



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SspI  
 1 CTAAATTGTAAGCGTTAAATAATTTGTTAAATTCGGTTAAATTTTGTAAATCAGCTCATTTTTTAACCAATAGGCGG  
 81 AAATCGGCAAAATCCCTTATAATCAAAAGAAATAGACCGAGATAGCGTTGAGTGTGTTCCAGTTTGGAAACAAGAGTCCA  
 161 CTATTAAAGAAGCTGGACTCCAACGTCAAAGCGGCAAAACCGTCTATCAGGGCGATGGCCCATACGTGAACCATCACC  
 241 CTAATCAAGTTTTTGGGTCGAGGTCCGTAAAGCACTAAATCGGAACCTTAAGGGAGCCCGGATTTAGAGCTTGAC  
 321 GGGAAACCGCGCAACGTGGCGAGAAAGGAAGCGAAGCGAAGGAGCGGGCGGTAGGGCGCTGGCAAGTGTAGCG  
 401 GTCACGCTCGCGTAACCAACACACCGCGGCTTAATGCGCGCTACAGGGCGGTCCCATTCGCCATTCAGGCTGCG  
 PvuII  
 481 CAACTGTTGGGAAGCGGATCGGTGGGGCTCTTCGCTATTACGCCAGCTGCCGAAGCGGATGCTGCAAGCGCAT  
 BssH II  
 561 TAAGTTGGTAACGCCAGGTTTTCCAGTCACGACGTGTAAACGACGCCAGTGAGCGCGGTAAATACGACTCACTA  
 SstI  
 641 TAGGGCAATTGGAGCTCCACCGCGGTGGCGGCGCTCTAGAGCAATTCCTTTGCCATAATTAATGAGGACTTAACCTG  
 XbaI  
 721 TGGAAATATTTTGATGTGGGAAGCTGTACTGTAAACCTGAGGTATTTGGGTAACTGCTATGTTAAACTTGCATTACAG  
 GGACACAAAAACATCATGAAAAATGGTGTGAAACCCCATCAAGGGTCAAAATTTTTCATTTTTTTCGCTTGGTGGCGAA  
 PstI  
 881 CCTTGGAGCTGCAGGTGTGTACCAAACTACAGGACCAATAATCCGTCTCAAACTGTAAACCCCAAAAAATGCTACAGT  
 HindII  
 961 TGACAGTCAGCAGATGAACACTGACCACAAGGCTGTTTTGGATAAGGATAATGCTTATCCAGTGGAGTGGCTGGGTTCCTG

FIG. 10B

SUBSTITUTE SHEET (RULE 26)

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1041 ATCCAAGTAAAAATGAAACACTAGATAATTTGGAACCTACACAGGTGGCGAAAAATGTGCCCTCCCTGTTTTGCCACATTACT  
HindII

1121 AACACAGCAACCACAGTCTTCTTGATGACGAGGGTGTGGGCCCTTGTGCAAAAGCTGACAGCTTGTATGTTTCTGCTGT  
HindII

1201 TGACATTGTGGGCTGTTTACCAACACTTCGGAACACAGCAGTGGAAAGGACTTCCCAGATAATTTTAAAAATTACCCCTTA  
HindII

1281 GAAAGCGGTCTGTGAAAAACCCCTACCCAATTTCCCTTTTGTAAAGTGACCTAATTAAACAGGACACAGAGGTGGAT  
BamHI

1361 GGCAGCCTATGATTGGAATGTCCCTCAAGTAGAGGAGTTAGGGTTTATGAGCACACAGAGGCTTCCTCGGGATCC  
BamHI

1441 AGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAAATGCAGTGAATAAAATGCTTTATTGTGAAATTT  
HindII

1521 GTGATGCTATTGCTTTATTTGTAACCATTAAGCTGCAATAAACAGTTAACAAACAATTCGATTCATTTTAIGTTTT  
HindII

1601 CAGCTTCAGGGGAGGTGTGGAGGTTTTTAAAGCAAGTAAACCTCTACAAAATGCGTATGGCTGATTATGATCTTTG  
HindII

FIG.10C

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FIG. 10D

1681 TGAAGGAACCTTACTTCGTGGTGTGACATAATTGGACAAACTACCTACAGAGATTAAAGGCCTAAGCTAAATATAAAA  
1761 TTTTAAAGTGATAAATGCTTAAACTACIGATTCTAATGTTTGTTGTAATTTTAGATCCAACCTATGGAACATGAIGAATG  
1841 GCAGCAGTGGTGGAAATGCCCTTAAATGAGGAAACCTGTTTTCGCTCAGAAGAAATGCCATCTAGTGAATGAGGCTACTG  
1921 CTGACTCTCAACATTCACICCTCCAAAAAAGAGAGAAAGGTAGAAGACCCCAAGGACTTTCCTTCAGAAATGCTAAGT  
2001 TTTTGGAGTCATGCTGTGTTTAGTAATAGAATCTTGGCTTGGCTATTTACACCACAAAGGAAAAAGCTGCACATGCT  
2081 ATACAAGAAAATTATGGAAAAATATTCGTAACTTTAAGTAGGCAATACAGTTATAATCAATACATACGTTTTTTC  
2161 TTACTCCACACAGGCATAGAGTGTCTGCTATTATAACTATGCTCAAAAATTTGTTACCTTTAGCTTTTAAATTTGTAAA  
2241 GGGGTTAATAAGGAATATTGAIGTATAGTCCCTTGACTAGAGATCGCCCTCCGGCGAATTTTCGCCATTCATCGGCTTA  
2321 TTATCACTTATTCAGCGGTAGCACCAGCGGTTTAAGGGCACCATAACTGCCTTAAAAAAATTACGCCCGCGCCCTGCCAC  
2401 TCATCGCAGTGCCTAGAACATAGTGGATCCCCCGGCTGCAGGAATTCGATGATCCICGCGCCCCCTACCCACCGTACTC  
337...GlyGlyGlu

XbaI SpeI BamHI SmaI PstI EcoRI

SUBSTITUTE SHEET (RULE 26)

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2481	GTCAATTCGAAGGGCATCGTTAAACATCTGCTCAAACTCGAAGTCGGCCATATCCAGAGCGCCGTAGCGGGCGGAGTCGT	
332	AspIleGlyLeuAlaAspThrPheMetGInGluPheGluPheAspAlaMetAspLeuAlaGlyTyrProAlaSerAspHis	
		SmaI
2561	GGCGGGTAAATCCCGGACCGCGGGAATCCCGGTCCCGCCAAACATGCCAGATCGTAATCGTCTAGCGCGTCGGCCTCGCGCATCGCGCC	
305	sProThrPheGlyProGlyProSerAspGlyAspGlyLeuMetAspLeuAspPheAspAspLeuAlaAspAlaHisAlaMet	HindII
		SacI
2641	ATCGCCACGTCTCGCGGTCTAAGTGGAGCTCGTCCCGCAGGCTGACATCGGTCCGGCGGGCGGTGACACAGTCTCGCGGT	
278	etAlaValAspGluGlyAspLeuHisLeuGluAspGlyLeuSerValAspThrProAlaThrSerLeuArgArgThr	SacII
		NdeI
2721	GTGTCCCGGGGAGAAAGGACAGCGCGGAGCCCGCCAGCCCGCTCTTCGGGGCGGTCTCGTCCGGCAGATCGACCA	
252	HisGlyAlaProLeuPheSerLeuArgProAlaAlaLeuGlyAlaGluProAlaAspAspAspProLeuAspLeuLe	
2801	GGCCCTCGATGGTAGACCGCTAATTGTTTTTCGTACCGCGCGGCTGACCGGACCCACTTTCACATTAACTTGTTTT	
225	uGlyGluIleThrSerGlyTyrAsnAsnLysThrArgAlaArgSerTyrAlaSerGlySerGluCysLysLeuGlnLysG	
		SmaI
2881	TCTAATCCGCATATGATCAATTCAAGCGCGAATAAGAAGGCTGGCTCTGCACCTTGGTGTACAAATAATTCGATAGCTTG	
198	luLeuGlyCysIleIleLeuGluLeuGlyPheLeuPheAlaProGluAlaGlyGlnHisAspPheLeuGluIleAlaGln	
2961	TCGTAATAATGGCGGCACTACTACAGTAGTAGGTCTTCCCTTCTTCTTAGCCACTTGATGCTCTTGATCTTCCAATA	
172	ArgLeuLeuProProMetSerAspThrThrProThrGluArgGluGluLysAlaValGlnHisGluGlnAspGluLeuVal	
3041	CGCAACCTAAAGTAAATGCCCCACAGCGCTGAGTGCATATAATGCATCTCTAGTGAATAAACCTTCTTGGCATATAAAG	
145	ICysGlyLeuThrPheHisGlyValAlaSerLeuAlaTyrLeuAlaAsnGluLeuSerPheGlyGlnGlnCysLeuPheA	
3121	GCTAATTGATTTTCGACAGTTTCATACTGTTTTCTGTAGGCGGTACCTAAATGTACTTTTTCCTCCATCGCGATGACT	
118	laLeuGlnAsnGluLeuThrGluTyrGlnLysGluThrProArgThrGlyLeuHisValLysAlaGlyAspArgHisSer	
3201	TAGTAAAGCACATCTAAACATTTTACCGTTATTACGTAAATAATCTTCCAGCTTTCCTCTCTAAAGGCGCAAAAGTCAG	
92	LeuLeuAlaCysArgPheSerLysAlaAsnAsnArgLeuPheAspGlnTrpSerGluGlyGluLeuProCysPheHisTh	
3281	TATGGTCCCTATCTAACATCTCAATGGCTAAGCGGTCCAGCAAGCCCGCTTATTTTACATGCCAATACAAATGTCAGCC	
65	rHisHisArgAspLeuMetGluIleAlaLeuAlaAspLeuLeuAlaArgLysAsnLysValHisTrpTyrLeuThrProG	

**FIG. 10E**

**SUBSTITUTE SHEET (RULE 26)**

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4561 TCGAAGCTCCCTCGTGGCTCTCTGTTCGACCTGCCGCTTACCGGATACCTGTCCGCCCTTTCICCCCTCCGGGAAGCG  
4641 TGGCGCTTTCICATAGCTACCGCTGAGGTATCTCAGTTCGGTGTAGGTGGTTCGGTCCCAAGCTGGGCTGTGTGCACGAA  
4721 CCCCCCGTTCAGCCCGACCGCTGCCCTTATCCGGTAACATCGCTTGAGTCCAAACCCGGTAAGACACGACTTATCGCC  
4801 ACTGGCAGCAGCAGTGGTAACAGGATTACGACGCGAGGTATGTAGGCGGTGCTACAGAGTTCCTTGAAGTGGTGGCCTA  
4881 ACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCCCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGC  
4961 TCTTGATCCGGCAAAACACCACCGCTGGTAGCGGTGGTTTTTTTGTTCGCAAGCAGAGATTACGCGCAGAAAAAAGG  
5041 ATCTCAAGAAGATCCTTTGATCTTTTCACGGGCTGACCGCTCAGTGAACGAAACACCTACGTTAAGGGAATTTGGTCA  
5121 TGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAATAATGAAGTTTTAAATCAATCTAAAGTATATATGAG  
5201 TAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAAGCACCTATCTCAGCGATCTGCTATTTTCGTTTCATCCATAGT  
5281 TGCCTGACTCCCGTGGTGTAGATAACTACGATACGGAGGGCTTACCATCTGGCCCGCAGTGTGCAATGATACCGGAG  
5361 ACCCAGGCTACCGGCTCCAGATTATACAGCAATAACCCAGCCAGCGGAGCGGAGCGCAGAGTGGTCTTGCACCT  
5441 TTATCCGGCTCCATCCAGTCTATTAAATGTTGGCGGGAAGCTAGAGTAAGTAGTTGCCAGTTAATAGTTTGGCAAGCT  
5521 TGTGGCATTTGCTACAGGATCGTGGTGTACCGCTGGTGGTATGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAA  
PvuI  
5601 GCGCAGTTACATGATCCCCCATGTGTGCAAAAAGCGGTAGCTCCTTCGGTCCCTCCGATCGTTGTCAGAAGTAAGTTG  
5681 GCGCAGTGTATATCATCATGTTATGGCAGCACTGCATAATCTCTTACGTGTCATGCCATCCGTAAGATGCTTTTCTGT  
ScaI  
5761 GACTGGTGAGTACTCAACCAAGTCATCTGAGAAATAGTGTATGCGGGCGACCGAGTTGCTCTTGCCCGCGCTCAATACGGG  
XmnI  
5841 ATAAACCGCGCCACATAGCAGAACTTTAAAAGTGTCTCATCATTTGGAACGTTCTTCGGGGCGAAAACTCTCAAGGATC  
5921 TTACCGCTGTGAGATCCAGTTCGATGTAAACCCACTCGTGCACCAACTGATCTTCAGCACTCTTTTACTTTCACCCAGGT  
6001 TTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCCGCAAAAAGCGGAATAAGGGCGACACCGGAAATGTTGAATACTCATAC  
SspI  
6081 TCTTCCTTTTTCATATTATTGAAGCATTTATCAGGGTTATGTCTCATGAGCGGATACATAATTGGAATGTATTAGAAA  
6161 AATAACAAATAGGGTTCGCGCCACATTTCCCGCAAAAGTCCAC

FIG.10G

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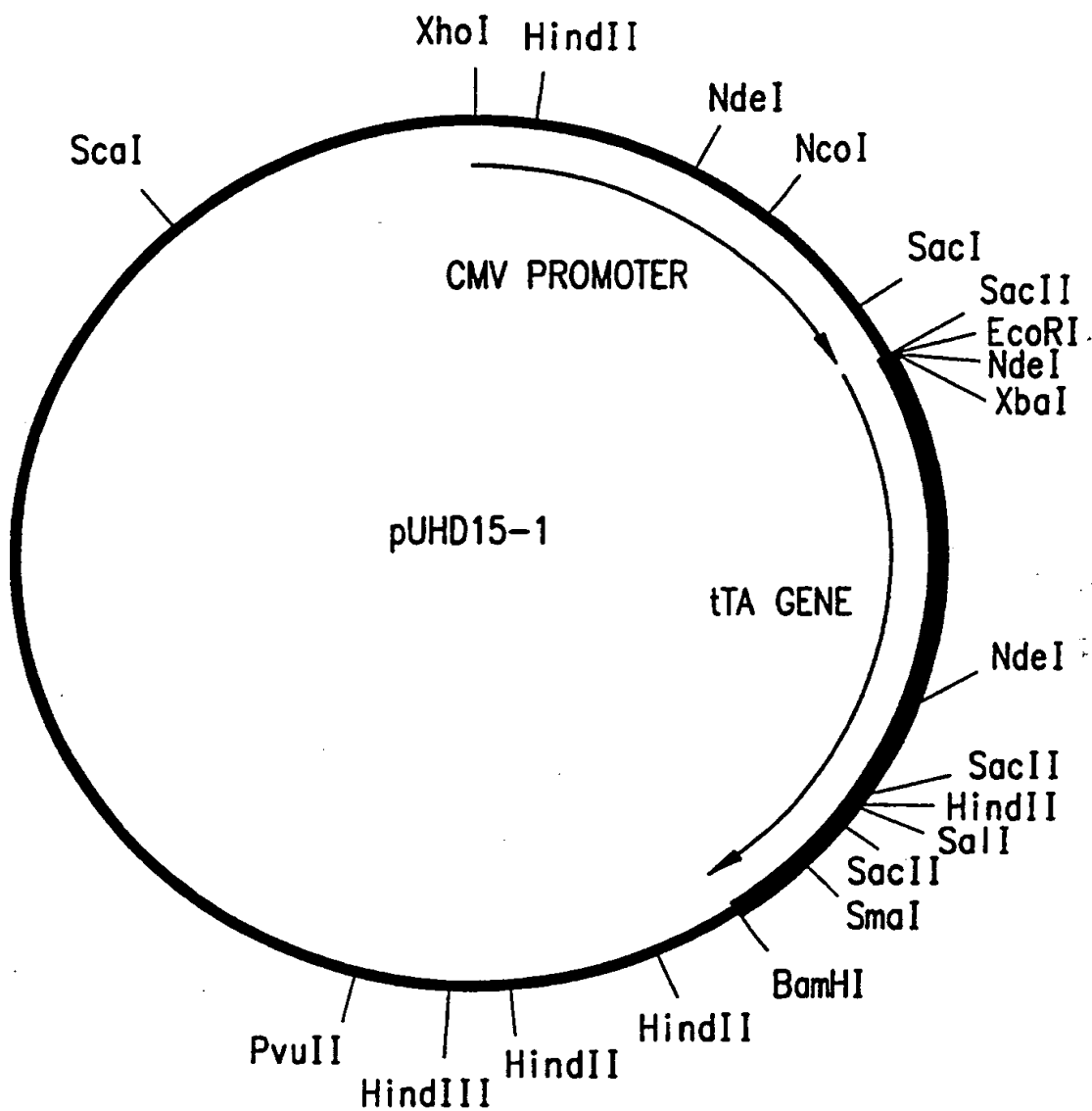


FIG.11A

SUBSTITUTE SHEET (RULE 26)

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XhoI  
 1 CTCGAGGAGC TTGGCCCAATT GCATACGTTG TATCCATATC ATAATATGTA CATTATATT GGCICATGIC  
 HindII  
 71 CAACATTACC GCCATGTTGA CATTGATTAT TGACTAGTTA TTAATAGTAA TCAATTACGG GGTCAATTAGT  
 141 TCATAGCCCA TATATGGAGT TCCGCGTTAC ATAATTACG GTAAATGGCC CGCTGGCTG ACCGCCCAAC  
 211 GACCCCGGCC CATTGACGTC AATAATGAGG TATGTTCCCA TAGTAAGGCC AATAGGGACT TTCCATTGAC  
 NdeI  
 281 GTCAATGGGT GGAGTATTTA CGCTAAACTG CCCACTTGGC AGTACATCAA GTGTATCATA TGCCAAGTAC  
 351 GCCCCTATT GACGTCAATG ACGGTAAATG GCCCGCCTGG CATTATGCCC AGTACATGAC CTTATGGGAC  
 NcoI  
 421 TTTCCTACTT GGCAGTACAT CTACGTATTA GTCATCGCTA TTACCATGGT GATGCGGTTT TGGCAGTACA  
 491 TCAATGGCGG TGGATAGCGG TTGACTCAC GGGGATTCC AAGTCTCCAC CCCATTGACG TCAATGGGAG  
 561 TTGTGTTTGG CACCAAATC AACGGGACTT TCCAAAATGT CGTAACAAC TCCGCCCAT T GACGCAAATG  
 SacI  
 631 GCGCGTAGGC GTGTACGGTG GGAGGTCTAT ATAAGCAGAG CTCGTTTAGT GAACCGTCAG ATCGCCCTGGA  
 SacII EcoRI  
 701 GACGCCATCC ACGCTGTTTT GACCTCCATA GAAGACACCG GGACCGATCC AGCCTCCGGG GCCCCGAATT  
 NdeI  
 771 CAT

FIG. 11B

SUBSTITUTE SHEET (RULE 26)

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XbaI

774 ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT AAC AGC GCA TTA GAG CTG CTT AAT GAG GTC  
 1•Met Ser Arg Leu Asp Lys Ser Lys Val Ile Asn Ser Ala Leu Glu Leu Leu Asn Glu Val  
 834 GGA ATC GAA GGT TTA ACA ACC CGT AAA CTC GCC CAG AAG CTA GGT GTA GAG CAG CCT ACA  
 21•Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln Lys Leu Gly Val Glu Gln Pro Thr  
 894 TTG TAT TGG CAT GTA AAA AAT AAG CGG GCT TTG CTC GAC GCC TTA GCC ATT GAG ATG TTA  
 41•Leu Tyr Trp His Val Lys Asn Lys Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu  
 954 GAT AGG CAC CAT ACT CAC TTT TGC CCT TTA GAA GGG GAA AGC TGG CAA GAT TTT TTA CGT  
 61•Asp Arg His His Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu Arg  
 1014 AAT AAC GCT AAA AGT TTT AGA TGT GCT TTA CTA AGT CAT CGC GAT GGA GCA AAA GTA CAT  
 81•Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp Gly Ala Lys Val His  
 1074 TTA GGT ACA CGG CCT ACA GAA AAA CAG TAT GAA ACT CTC GAA AAT CAA TTA GCC TTT TTA  
 101•Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu Thr Leu Glu Asn Gln Leu Ala Phe Leu  
 1134 TGC CAA CAA GGT TTT TCA CTA GAG AAT GCA TTA TAT GCA CTC AGC GCT GTG GGG CAT TTT  
 121•Cys Gln Gln Gly Phe Ser Leu Glu Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe  
 1194 ACT TTA GGT TGC GTA TTG GAA GAT CAA GAG CAT CAA GTC GCT AAA GAA GAA AGG GAA ACA  
 141•Thr Leu Gly Cys Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr  
 1254 CCT ACT ACT GAT AGT ATG CCG CCA TTA TTA CGA CAA GCT ATC GAA TTA TTT GAT CAC CAA  
 161•Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu Phe Asp His Gln  
 NdeI

1314 GGT GCA GAG CCA GCC TTC TTA TTC GGC CTT GAA TTG ATC ATA TGC GGA TTA GAA AAA CAA  
 181•Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu Ile Ile Cys Gly Leu Glu Lys Gln

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FIG. 11C

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1374 CTT AAA TGT GAA AGT GGG TCC GCG TAC AGC CGC GCG CGT ACG AAA AAC AAT TAC GGG TCT
201▶Leu Lys Cys Glu Ser Gly Ser Ala Tyr Ser Arg Ala Arg Thr Lys Asn Asn Tyr Gly Ser
1434 ACC ATC GAG GGC CTG GAT CTC CCG GAC GAC GAC GCG CCC GAA GAG GCG GCG CTG GCG
221▶Thr Ile Glu Gly Leu Asp Leu Pro Asp Ala Pro Glu Glu Ala Gly Leu Ala
                                     HindII
1494 GCT CCG CGC CTG TCC TTT CTC CCC GCG GGA CAC ACG CGC AGA CTG TCG ACG GCC CCC CCG
241▶Ala Pro Arg Leu Ser Phe Leu Pro Ala Gly His Thr Arg Arg Leu Ser Thr Ala Pro Pro
                                     SacII
1554 ACC GAT GTC AGC CTG GGG GAC GAG CTC CAC TTA GAC GGC GAG GAC GTG GCG ATG GCG CAT
261▶Thr Asp Val Ser Leu Gly Asp Glu Leu His Leu Asp Gly Glu Asp Val Ala Met Ala His
                                     SmaI
1614 GCC GAC GCG CTA GAC GAT TTC GAT CTG GAC ATG TTG GGG GAC GGG GAT TCC CCG GGT CCG
281▶Ala Asp Ala Leu Asp Phe Asp Leu Asp Met Leu Gly Asp Gly Asp Ser Pro Gly Pro
1674 GGA TTT ACC CCC CAC GAC TCC GCC CCC TAC GGC GCT CTG GAT ATG GCC GAC TTC GAG TTT
301▶Gly Phe Thr Pro His Asp Ser Ala Pro Tyr Gly Ala Leu Asp Met Ala Asp Phe Glu Phe
1734 GAG CAG ATG TTT ACC GAT GCC CTT GGA ATT GAC GAG TAC GGT GGG TAG
321▶Glu Gln Met Phe Thr Asp Ala Leu Gly Ile Asp Glu Tyr Gly Gly ...
                                     BamHI
1782 GGGGCGCGAG GATCCAGACA TGATAAGATA CATTGATGAG TTGGACAAA CCACAAC TAG AATGCAGTGA
1852 AAAAAATGCT TTATTGTGA AATTGTGAT GCTATTGCTT TATTGTAAC CATTATAAGC TGCAATAAAC
                                     HindII
1922 AAGTTAACAA CAACAATTGC ATTCATTTTA TGTTTCAGGT TCAGGGGGAG GTGTGGGAGG TTTTAAAG
1992 CAAGTAAAC CTCTACAAAT GTGGTATGGC TGATTATGAT CCTGCAAGCC TCGTGTCTG GCCGGACCAC
2062 GCTATCTGTG CAAGGTCCCG GGACGCGCGC TCCATGAGCA GAGCGCCCGC CGCCGAGGCA AGACTCGGGC

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FIG. 11D

SUBSTITUTE SHEET (RULE 26)

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HindII  
2132 GCGGCCCTGC CCGTCCCACC AGGTCAACAG GCGGTAACCG GCCTCTTCAT CGGGAATGCG GCGGACCTTC  
HindIII  
2202 AGCATCGCG GCATGTCCCC TGGCGGACGG GAAGTATCAG CTCGACCAAG CTTGGCGAGA TTTTCAGGAG  
2272 CTAAGGAAGC TAAATGGAG AAAAAAATCA CTGGATATAC CACCGTTGAT ATATCCCAAT GGCAATCGTAA  
PvuII  
2342 AGAACAATTT GAGGCATTTT AGTCAGTTGC TCAATGTACC TATAACCAGA CCGTTCAGCT GCATTAATGA  
2412 ATCGGCCAAC GCGCGGGGAG AGGCGGTTTG CGTATTGGG GCCTTCCGC TTCTTCGCTC ACTGACTCGC  
2482 TGGGCTCGGT CGTTCGGCTG CGGCGAGCGG TATCAGCTCA CTCAAAGCGG GTAATACGGT TATCCACAGA  
2552 ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC CAGCAAAAGG CCAGGAACCG TAAAAAGGCC  
2622 GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCTGACG AGCATCACAA AAATCGACGC TCAAGTCAGA  
2692 GGTGGGGAAC CCGACAGGA CTATAAGAT ACCAGGCGTT TCCCCCTGGA AGTCCCTCG TCGGCTCTCC  
2762 TGTTCGACC CTGCGCTTA CCGGATACCT GTCCGCCCTT CTCCCTTCGG GAAGCGTGGC GCCTTCTCAA  
2832 TGCTCAGCT GTAGGTATCT CAGTTCGGTG TAGGTGTTT GCTCCAAGT GGGCTGTGTG CACGAACCCC  
2902 CCGTTCAGCC CGACCGCTGC GCCTTATCCG GTAACATCG TCTTGAGTCC AACCCGGTAA GACACGACTT  
2972 ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA GCGAGGTATG TAGGCGGTGC TACAGAGTTC  
3042 TTGAAGTGGT GGCCTAACTA CCGCTACACT AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG  
3112 TTACCTTCGG AAAAAGAGTT GGTAGCTCTT GATCCGGCAA ACAAAACCACC GCTGGTAGCG GTGGTTTTT  
3182 TGTITGCAAG CAGCAGATTA CGCGCAGAAA AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG  
3252 TCTGACGCTC AGTGGAACGA AAATCAGCT TAAGGGATTT TGGTCATGAG ATTATCAAAA AGGATCTTCA  
3322 CCTAGATCCT TTTAAATTAA AAATGAAGTT TTAATCAAT CTAAAGTATA TAAGATAA CTGGTCTGA  
3392 CAGTTACCAA TGCTTAATCA GTGAGGCACC TATCTCAGCG ATCTGTCTAT TTCGTTTCAT CATAGTTGCC  
3462 TGAATCCCCG TCGTGTAGAT AACTACGATA CCGGAGGGCT TACCATCTGG CCCCAGTGT GCAATGATAC  
3532 CCGGAGACCC ACGCTCACC GCTCCAGATT TATCAGCAAT AAACAGCCA GCCGGAAGGG CCGAGCGCAG  
3602 AAGTGGTCTT GCAACTTTAT CCGCTCCAT CCAGTCTATT AATTGTTGCC GGAAGCTAG AGTAAGTAGT  
3672 TCGCCAGTTA ATAGTTTGGC CAACGTGTT GCCATTGCTA CAGGCATCGT GGTGTCACGC TCGTGGTTTG

FIG. 11E

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3742 GTATGGCTTC ATTGAGCTCC GGTTCCTCAAC GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA  
3812 AGCGGTTAGC TCCTTCGGTC CTCGATCGT TGTGAGAGT AAGTTGGCCG CAGTGTATC ACTCATGGTT  
ScaI  
3882 ATGGCAGCAC TGCATAATTC TCTTACTGTC ATGCCATCCG TAAGATGCTT TTCGTGACT GGTGAGTACT  
3952 CAACCAAGTC ATTCTGAGAA TAGTGATGC GCGGACCGAG TTGCTCTTGC CCGGCGTCAA TACGGGATAA  
4022 TACCGCGCCA CATAGCAGAA CTTTAAAGT GCTCATCATT GGAAAACGTT CTTCGGGGCG AAACCTCTCA  
4092 AGGATCTTAC CGCTGTTGAG ATCCAGTTCC ATGTAACCCA CTCGTGCACC CAACIGATCT TCAGCATCTT  
4162 TTACTTTCAC CAGCGTTTCT GGGTGAGCAA AACACGGGAG GCAAAATGCC GCAAAAAAGG GAATAAGGCG  
4232 GACACGGAAA TGTGAATAC TCATACICTT CCTTTTTCAA TATTATTGAA GCATTTAICA GGGTTATTGT  
4302 CTCATGAGCG GATACATAAT TGAATGTATT TAGAAAAATA AACAAATAGG GGTTCGCGCG ACATTTCGCC  
4372 GAAAAGTGGC ACCTGACGTC TAAGAAACCA TTATTATCAT GACATTAAACC TATAAAAAATA GCGGTATCAC  
4442 GAGGCCCTTT CGTC

FIG. 11F

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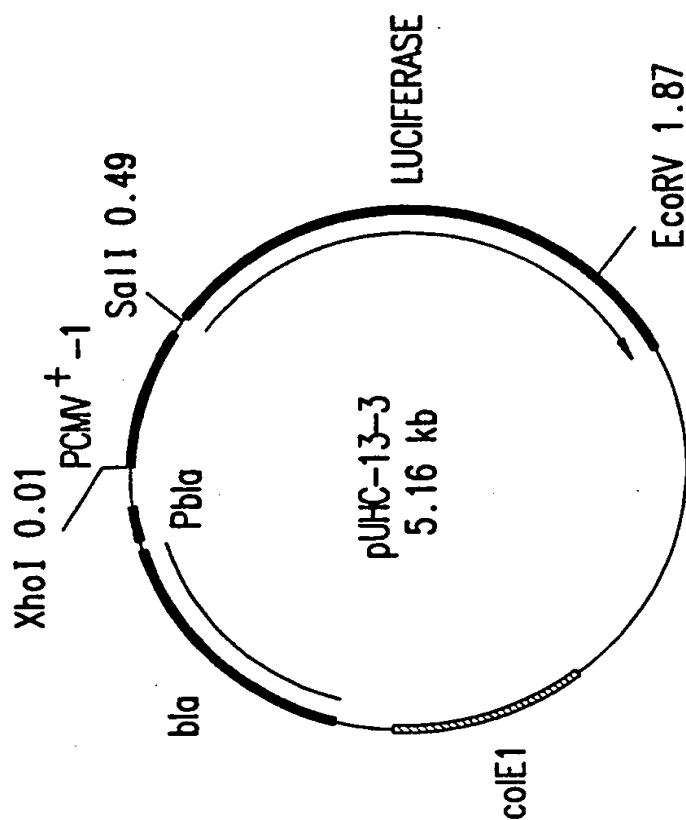


FIG.12A

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EcoRI XhoI  
 1 GAATTCCTCG AGTTTACCAC TCCCTATCAG TGATAGAGAA AAGTGAAAGT CGAGTTTACC ACTCCCTAIC  
 71 AGTGATAGAG AAAAGTGAAA GTCGAGTTTA CCACTCCCTA TCAGTGATAG AGAAAAGTGA AAGTCGAGTT  
 141 TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCGAG TTTACCACATC CCTATCAGIG ATAGAGAAAA  
 211 GTGAAAGTCG AGTTTACCAC TCCCTATCAG TGATAGAGAA AAGTGAAAGT CGAGTTTACC ACTCCCTAIC  
 281 AGTGATAGAG AAAAGTGAAA GTCGAGCTCG GTACCCGGGT CGAGTAGGCG TGTACGGTGG GAGGCCTATA  
 351 TAAGCAGAGC TCGTTTAGTG AACCGTCAGA TCGCCTGGAG ACGCCATCCA CGCTGTTTGG ACCTCCATAG  
 421 AAGACACCGG GACCGATCCA GCCTCCGGG CCCCGAATTC GAGCTCGGTA CCCGGGGGATC CTCTAGAGTC  
 491 GACCTGCAGG C  
 HindIII  
 502 ATG CAA GCT TGG CAT TCC GGT ACT GTT GGT AAA ATG GAA GAC GCC AAA AAC ATA AAG AAA  
 1 Met Gln Ala Trp His Ser Gly Thr Val Gly Lys Met Glu Asp Ala Lys Asn Ile Lys Lys  
 XbaI  
 562 GGC CCG GCG CCA TTC TAT CCT CTA GAG GAT GGA ACC GCT GGA GAG CAA CTG GAT AAG CCT  
 21 Gly Pro Ala Pro Phe Tyr Pro Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu Asp Lys Pro  
 622 ATG AAG AGA TAC GCC CTG GTT CCT GGA ACA ATT GCT TTT ACA GAT GCA CAT ATC GAG GTG  
 41 Met Lys Arg Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu Val  
 682 AAC ATC ACG TAC GCG GAA TAC TTC GAA ATG TCC GTT CGG TTG GCA GAA GCT GTG AAA CGA  
 61 Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala Glu Ala Val Lys Arg

FIG. 12B

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742 TAT GGG CTG AAT ACA AAT CAC AGA ATC GTC GTA TTC AGT GAA AAC TCT CTT CAA TTC TTT  
 81▶Tyr Gly Leu-Asn Thr Asn His Arg Ile Val Val Phe Ser Glu Asn Ser Leu Gln Phe Phe  
 802 ATG CCG GTG TTG GGC GCG TTA TTT ATC GGA GTT GCA GTT GCG CCC GCG AAC GAC ATT TAT  
 101▶Met Pro Val Leu Gly Ala Leu Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr  
 862 AAT GAA CGT GAA TTG CTC AAC AGT ATG AAC ATT TCG CAG CCT AAC GTA GTG TTG GTT TCC  
 121▶Asn Glu Arg Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Asn Val Val Leu Val Ser  
 922 AAA AAG GGG TTG CAA AAA ATT TTG AAC GTG CAA AAA AAA TTA CCA ATA ATC CAG AAA ATT  
 141▶Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro Ile Ile Gln Lys Ile  
 982 ATT ATC ATG GAT CTC AAA ACG GAT TAC CAG GGA TTT CAG TCG ATG TAC ACG TTC GTC ACA  
 161▶Ile Ile Met Asp Leu Lys Thr Asp Tyr Gln Gly Phe Gln Ser Met Tyr Thr Phe Val Thr  
 1042 TCT CAT CTA CCT CCC GGT TTT AAT GAA TAC GAT TTT GTA CCA GAG TCC TTT GAT CGT GAC  
 181▶Ser His Leu Pro Pro Gly Phe Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp  
 EcoRI  
 1102 AAA ACA ATT GCA CTG ATA ATG AAT TCC TCT GGA TCT ACT GGG TTA CCT AAG GGT GTG GCC  
 201▶Lys Thr Ile Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val Ala  
 1162 CTT CCG CAT AGA ACT GCC TGC GTC AGA TTC TCG CAT GCC AGA GAT CCT ATT TTT GGC AAT  
 221▶Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp Pro Ile Phe Gly Asn  
 1222 CAA ATC ATT CCG GAT ACT GCG ATT TTA AGT GTT GTT CCA TTC CAT CAC GGT TTT GGA ATG  
 241▶Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val Val Pro Phe His His Gly Phe Gly Met  
 1282 TTT ACT ACA CTC GGA TAT TTG ATA TGT GGA TTT CGA GTC GTC TTA ATG TAT AGA TTT GAA  
 261▶Phe Thr Leu Gly Tyr Leu Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu  
 1342 GAA GAG CTG TTT TTA CGA TCC CTT CAG GAT TAC AAA ATT CAA AGT GCG TTG CTA GTA CCA  
 281▶Glu Glu Leu Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val Pro  
 1402 ACC CTA TTT TCA TTC TTC GCC AAA AGC ACT CTG ATT GAC AAA TAC GAT TTA TCT AAT TTA  
 301▶Thr Leu Phe Ser Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr Asp Leu Ser Asn Leu

FIG. 12C

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1462 CAC GAA ATT GCT TCT GGG GGC GCA CCT CTT TCG AAA GAA GTC GGG GAA GCG GTT GCA AAA  
 321►His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser Lys Glu Val Gly Glu Ala Val Ala Lys  
 1522 CGC TTC CAT CTT CCA GGG ATA CGA CAA GGA TAT GGG CTC ACT GAG ACT ACA TCA GCT ATT  
 341►Arg Phe His Leu Pro Gly Ile Arg Gln Gly Tyr Gly Leu Thr Glu Thr Ser Ala Ile  
 1582 CTG ATT ACA CCC GAG GGG GAT GAT AAA CCG GGC GCC GTC GGT AAA GTT GTT CCA TTT TTT  
 361►Leu Ile Thr Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe Phe  
 1642 GAA GCG AAG GTT GTG GAT CTG GAT ACC GGG AAA ACG CTG GGC GTT AAT CAG AGA GGC GAA  
 381►Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val Asn Gln Arg Gly Glu  
 1702 TTA TGT GTC AGA GGA CCT ATG ATG TCC GGT TAT GTA AAC AAT CCG CAA GCG ACC AAC  
 401►Leu Cys Val Arg Gly Pro Met Met Ser Gly Tyr Val Asn Asn Pro Gln Ala Thr Asn  
 1762 GCC TTG ATT GAC AAG GAT GGA TGG CTA CAT TCT GGA GAC ATA GCT TAC TGG GAC GAA GAC  
 421►Ala Leu Ile Asp Lys Asp Gly Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp  
 1822 GAA CAC TTC TTC ATA GTT GAC CGC TTG AAG TCT TTA ATT AAA TAC AAA GGA TAT CAG GTG  
 441►Glu His Phe Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln Val  
 1882 GCC CCC GCT GAA TTG GAA TCG ATA TTG TTA CAA CAC CCC AAC ATC TTC GAC GCG GGC GTG  
 461►Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile Phe Asp Ala Gly Val  
 1942 GCA GGT CTT CCC GAC GAT GAC GCC GGT GAA CTT CCC GCC GCT GTT GTT TTG GAG CAC  
 481►Ala Gly Leu Pro Asp Asp Ala Gly Glu Leu Pro Ala Ala Val Val Val Leu Glu His  
 2002 GGA AAG ACG ATG ACG GAA AAA GAG ATC GTG GAT TAC GTC GCC AGT CAA GTA ACA ACC GCC  
 501►Gly Lys Thr Met Thr Glu Lys Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala  
 2062 AAA AAG TTG CGC GGA GGA GTT GTG TTT GTG GAC GAA GTA CCG AAA GGT CTT ACC GGA AAA  
 521►Lys Lys Leu Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly Lys  
 2122 CTC GAC GCA AGA AAA ACT AGA GAG ATC CTC ATA AAG GCC AAG GGC GGA AAG TCC AAA  
 541►Leu Asp Ala Arg Lys Thr Arg Glu Ile Leu Ile Lys Ala Lys Lys Gly Lys Ser Lys  
 2182 TTG TAA  
 561►Leu ...

FIG. 12D

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XbaI  
2188 AATGTAAC TGATCAGCGA TGACGAAATT CTAGCTATT GTAATGACTC TAGAGGATCT TTGIGAAGGA  
2258 ACCTTACTC TGTTGGTGTGA CATAATTGGA CAAACTACCT ACAGAGATT AAAGCTCTAA GGTAATATA  
2328 AAATTTTAA GTGTATAATG TGTAAACTA CTGATTCTAA TTGTTGTGT ATTTAGATT CCAACCTATG  
2398 GAACTGATGA ATGGAGCAG TGGTGGAAATG CCTTAATGA GGAACCTCG TTTTGCICAG AAGAAATGCC  
2468 ATCTAGTGAT GATGAGGCTA CTGCTGACTC TCAACATCT ACICCTCCAA AAAAGAAGAG AAAGGTAGAA  
2538 GACCCCAAGG ACTTCCCTTC AGAATTGCTA AGTTTTTGA GTCATGCTGT GTTTAGTAAT AGAACTCTTG  
2608 CTTGCTTTC TATTACACC ACAAGGAAA AAGCTGCACT GCTATACAAG AAAATTATGG AAAAATATTC  
2678 TGTAAACCTT ATAAGTAGGC ATAACAGTTA TAATCATAAC ATACTGTTT TICTTACTCC ACACAGGCAT  
2748 AGAGTGTCTG CTATTATAA CTAIGCTCAA AAATIGTGA CCITTAGCTT TTTAATTGT AAAGGGTTA  
2818 ATAAGGAATA TTTGATGAT AGTGCCTTGA TCATAATCAG CCATACCACA TTTGTAGAGG TTTTACTTGC  
2888 TTTAAAAAAC CTCACACACC TCCCCCTGAA CCTGAAACAT AAAATGAATG CAATTGTTGT TGTAACTTG  
2958 TTTATTGCAG CTTATAATGG TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA GCATTTTTTT  
XbaI  
3028 CACTGCATC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGCCTCT AGAGCTGCAT  
3098 TAATGAATCG GCCAACGCGC GGGGAGAGGC GGTITGCGTA TTGGGCGCTC TTCCGCTTCC TCGTCACTG  
3168 ACTCGCTGCG CTCGGTCTGT CCGGTGCGC GAGCGGTATC AGCTCACTCA AAGCGGTAA TACGGTTATC  
3238 CACAGAATCA GGGGATAACG CAGGAAGAA CATGTGAGCA AAAGGCCAGC AAAGGCCAG GAACCGTAA  
3308 AAGGCCCGT TGTGGCGT TTTCATAGG CTCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA  
3378 GTCAGAGGTG GCGAAACCG ACAGGACTAT AAAGATACCA GCGTTTCCC CCTGGAAGCT CCCTCGTGCG  
3448 CTCTCCTGTT CCGACCTGC CGCTTACCGG ATACCTGTCC GCCTTCTCC CTTGGGAAG CGTGGCGCTT  
3518 TCTCAATGCT CACGCTGTAG GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC TGTGTGCAG  
3588 AACCCCGCT TCAGCCCGAC CGCTGCGCT TATCCGGTAA CTATCGTCTT GAGTCCAACC CGGTAAGACA  
3658 CGACTTATCG CCACTGGCAG CAGCCACTGG TAACAGGATT AGCAGAGCGA GGTATGTAGG CCGTGTCTACA  
3728 GAGTCTTGA AGTGGTGGC TAACTACGGC TAACTAGAA GGACAGTATT TGGTATCTGC GCTCTGCTGA  
3798 AGCCAGTTAC CTTCCGAAA AGAGTTGGTA GCTCTTGATC CGGCAACAA ACCACCGCTG GTAGCGGTGG

FIG. 12E

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3868 TTTTITTTGTT TGAAGCAGC AGATTACGG CAGAAAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT  
3938 ACGGGTCTG ACGCTCAGTG GAACGAAAC TCACGTTAAG GGATTTTGGT CATGAGATTA TCAAAAAAGGA  
4008 TCTTCAACCTA GATCCTTTTA AATTAAAAAT GAAGTTTTAA ATCAATCTAA AGTATATAAG AGTAAACTTG  
4078 GTCTGACAGT TACCAATGCT TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTTG TTCAATCCATA  
4148 GTTGCCTGAC TCCCGTCTGT GTAGATAACT ACGATACGGG AGGGCTTACC ATCTGGCCCC AGTGTGCAA  
4218 TGATACCGCG AGACCCACGC TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG GAAGGGCCGA  
4288 GCGCAGAAGT GGTCTGTCAA CTTTATCCGC CTCATCCAG TCTATTAATT GTTGGCCGGA AGCTAGAGTA  
4358 AGTAGTTTCG CAGTTAATAG TTGCGCAAC GTTGTGCGA TTGCTACAGG CATCGTGGTG TCACGCTCGT  
4428 CGTTTGGTAT GGCTTCATTC AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC CCATGTTGTG  
4498 CAAAAAAGCG GTTAGCTCCT TCGGTCCCTCC GATCGTTGTC AGAAGTAAAT TGGCCGCAGT GTTATCACTC  
4568 ATGGTTATGG CAGCACIGCA TAATTCCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG  
4638 AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC TCTTGGCCCG CGTCAATACG  
4708 GGATAATACC GCGCCACATA GCAGAACTTT AAAAGTGTCT ATCATTTGAA AACGTTCTTC GGGGCGAAAA  
4778 CTCTCAAGGA TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCACTCG TGCACCCCAAC TGATCTTCAG  
4848 CATCTTTTAC TTTACCCAGC GTTCTGGGT GAGCAAAAAAC AGGAAGGCAA AATGCCGCAA AAAAGGGAAT  
4918 AAGGGCGACA CGGAAATGTT GAATACTCAT ACTCTTCTT TTTCATATTT ATTTGAAGCAT TTATCAGGGT  
4988 TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA AATAGGGGTT CCGCGCACAT  
5058 TTCCCCGAAA AGTGCCACCT GACGTGACGT CTAGAAACC ATTATTATCA TGACATTAACT CTATAAAAAT  
5128 AGCGGTATCA CGAGGCCCTT TCGTCTTCAA

FIG. 12F

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/10109

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/63 C12N15/67 C12N15/85 C12N15/62 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROC NATL ACAD SCI U S A, JUL 3 1995, 92 (14) P6522-6, UNITED STATES, XP002015265 SHOCKETT P ET AL: "A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice." see the whole document ---	1-20
P,X	WO,A,96 01313 (BUJARD HERMANN ;GOSSEN MANFRED (US)) 18 January 1996 see page 4-5; figure 9 --- -/--	1-20

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

7 October 1996

Date of mailing of the international search report

18. 10. 96

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Espen, J

## INTERNATIONAL SEARCH REPORT

International Application No.

PCI/US 96/10109

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PROC NATL ACAD SCI U S A, MAY 28 1996, 93 (11) P5185-90, UNITED STATES, XP002015266 HOFMANN A ET AL: "Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette [see comments]" see figure 1 ---	1-20
Y	DE,A,39 34 454 (MERCK PATENT GMBH) 18 April 1991 see page 6, line 27 - line 37; figures 3,4 ---	1-20
Y	PROC NATL ACAD SCI U S A, JUN 15 1992, 89 (12) P5547-51, UNITED STATES, XP000564458 GOSSEN M ET AL: "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters." see the whole document -----	1-20

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/10109

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9601313	18-01-96	AU-A- 3092395	25-01-96
DE-A-3934454	18-04-91	NONE	

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